Genetic Characterization of the Interaction between Italian Ryegrass (*Lolium multiflorum* Lam.) and *Xanthomonas translucens* pv. *graminis*

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Summary

Grasslands are among the largest ecosystems in the world and represent the main source of natural low-cost feed for ruminants. Ryegrasses are major components of such grassland systems and Italian ryegrass (*Lolium multiflorum* Lam.) is a very widely used plant species for intensive hay and silage production. *Xanthomonas translucens* pv. *graminis* (*Xtg*) is a γ-proteobacterial plant pathogen and causes bacterial wilt on a number of forage grass species including the genera *Agrostis*, *Alopecurus*, *Bromus*, *Dactylis*, *Festuca*, *Lolium*, *Phleum*, *Poa* and *Trisetum.* The pathogen spreads through wounds and breaches caused when mowing with contaminated equipment. Compared to all plant species included in the host range of *Xtg*, *L. multiflorum* is particularly susceptible and the disease can lead to complete yield losses in pure and mixed stands depending on cultivar susceptibility. Breeding for resistant cultivars by means of phenotypic recurrent selection is the most practicable means of disease control and has lead to the development of cultivars with improved resistance to bacterial wilt. Nevertheless, due to the out-breeding reproduction mode of *L. multiflorum* and the population-based breeding schemes used for breeding forage grasses, further progress is difficult to obtain and highly susceptible individuals still occur in advanced breeding material. Since resistance mechanisms of *L. multiflorum* and virulence mechanisms of *Xtg* are largely unknown, the main objective of this thesis was to understand in more detail the interaction of *Xtg* with *L. multiflorum*. The application of genomic approaches combined with transcriptome analyses enables the identification of genes and genomic regions that affect host resistance and pathogen virulence.

The first aim of this thesis was to elucidate the existence of race-specificity in the *L. multiflorum-Xtg* interaction and to identify genomic regions that contribute to *Xtg* resistance using marker-trait associations (chapter 2). No major race-specific interactions were found in 62 *L. multiflorum* genotypes infected with six *Xtg* isolates. Molecular marker-resistance associations revealed one simple sequence repeat (SSR) marker on linkage group (LG) 5 to be significantly associated with bacterial wilt resistance using the same plant genotypes across all six bacterial isolates. The identified SSR marker explained up to 37.4% of the total variance of area under the disease progress curve (AUDPC) values. The second aim of this thesis was to identify candidate genes in *L. multiflorum* for *Xtg* resistance by means of cross-species hybridization to a cDNA microarray of perennial ryegrass (*L. perenne*) using a resistant and a susceptible *L. multiflorum* genotype (chapter 3). Major transcriptomic differences were observed between the susceptible and the resistant *L. multiflorum* genotype

especially after *Xtg* infection. Transcriptome analyses of solely the resistant genotype after *Xtg* infection revealed a number of promising candidate genes for bacterial wilt resistance such as the low silicon transporter (Lsi1) and the germin-like protein 6 (GLP6).

To investigate bacterial factors which might be the target of plant resistance mechanisms, we screened for known virulence genes in *Xtg*. Phytopathogenic *Xanthomonas* spp. have many virulence associated characteristics in common; nevertheless, the symptoms caused and the significance of these virulence factors for the interaction with their hosts may differ greatly. However, the type III secretion system (T3SS) represents one of the major virulence factors found in most *Xanthomonas* spp. and mediates the delivery of disease promoting effector proteins and avirulence products into their host. Screening *Xtg* for genes encoding T3SS components was performed using conserved primers based on publicly available sequences of *Xanthomonas* spp. Despite high conservation among the sequences encoding T3SS components of *Xanthomonas* spp., this approach was only successful for the *hrpG* gene of *Xtg*. After amplification and sequencing of the *hrpG* gene, a mutant deficient of the *hrpG* gene was obtained by means of double homologous recombination (chapter 4). Virulence of the *ΔhrpG* mutant was significantly reduced when infecting *L. multiflorum* plants. However, *in planta* analyses revealed that the *ΔhrpG* mutant was still able to survive and multiply inside its host.

Due to distant phylogenetic relationships between *Xtg* and the other sequenced *Xanthomonas* spp. and in order to elucidate the existence of a T3SS and other virulence mechanisms of *Xtg*, the whole genome of the *Xtg*29 isolate was sequenced by means of 454 sequencing. This shotgun sequencing approach enabled a preliminary analysis of the gene cluster encoding components of the T3SS, genes encoding effectors and other bacterial virulence factors (chapter 5) and has revealed an entire T3SS gene cluster and genes encoding 22 putative effector proteins. Additional functional characterization of the genes encoding virulence factors and effector proteins may enable the identification of resistance mechanisms inside the host plants.

Overall, the data presented in this thesis provides a more comprehensive knowledge of the interaction between *Xtg* and *L. multiflorum* and represent a valuable basis for the development of tools for marker-assisted selection (MAS) in the future.

Zusammenfassung

Grasland ist eines der grössten und wichtigsten Ökosysteme der Welt und stellt die dominanteste Futterquelle für die Milch- und Fleischproduktion dar. Raigräser (*Lolium* spp.) sind wichtige Bestandteile solcher Graslandsysteme und vor allem das Italienische Raigras (*Lolium multiflorum*) wird häufig für die intensive Heu- und Silageproduktion angebaut. Bakterienwelke ist eine schwerwiegende Krankheit, die durch *Xanthomonas translucens* pv. *graminis* (*Xtg*), ein γ-Proteobakterium, hervorgerufen wird. Bakterienwelke tritt neben *L. multiflorum* auch bei anderen Futtergräsern auf und je nach Anfälligkeit der Gräserart kann die Krankheit zum totalen Ertragsausfall führen. Deshalb stellt die Züchtung von resistenten Sorten die wichtigste und effizienteste Kontrollmassnahme gegen Bakterienwelke dar. Mit phänotypischer Selektion sind bis heute bereits gute Erfolge erzielt worden und relativ resistente Raigras Sorten konnten auf den Markt gebracht werden. Trotzdem treten immer noch sehr anfällige Pflanzen in fortgeschrittenem Zuchtmaterial auf. Um weitere Züchtungsfortschritte erzielen zu können, ist deshalb ein umfangreicheres Wissen über die Quelle der Krankheitsresistenz und Virulenzmechanismen der Bakterien eine der wichtigsten Voraussetzungen. Deshalb war das Hauptziel dieser Arbeit, die Interaktion von *L. multiflorum* und *Xtg* besser zu charakterisieren. Um dieses Ziel zu erreichen, können genomische Analysen und Genexpressionsstudien wichtige Erkenntnisse liefern, die dann zur Identifizierung von Resistenzgenen und wichtigen Genomregionen führen können.

Das erste spezifische Ziel stellte die Aufklärung der Existenz von Rassenspezifität in der *L. multiflorum-Xtg* Interaktion dar. Zudem wurden molekulare Marker untersucht, die mit Bakterienwelke Resistenz assoziiert sind (Kapitel 2). Es konnte keine Rassenspezifität gefunden werden in den 62 *L. multiflorum* Genotypen, die mit sechs verschiedenen *Xtg* Isolaten infiziert wurden. Die Analyse mit molekularen Markern zeigte, dass innerhalb derselben Genotypen ein Mikrosatelliten Marker (simple sequence repeat: SSR) auf der Kopplungsgruppe 5 signifikant mit Bakterienwelke-Resistenz assoziiert war. Der SSR Marker erklärte bis zu 37.4% der Gesamtvarianz für die Werte der Fläche unter der Krankheitskurve (area under the disease progress curve: AUDPC). Das zweite spezifische Ziel dieser Studie war, Kandidatengene für *Xtg* Resistenz in *L. multiflorum* zu identifizieren mittels einer Genexpressionsanalyse (Kapitel 3). Dazu wurde ein cDNA Mircorarray verwendet, der ursprünglich für Genexpressionsanalysen mit Englischem Raigras (*L. perenne*) entwickelt worden war. Für die Genexpressionsstudie wurden ein sehr anfälliger und ein resistenter *L. multiflorum* Genotyp verwendet. Genexpressionsunterschiede nach Infektion mit *Xtg* und nach Kontrollbehandlung ohne *Xtg* wurden zwischen den Genotypen ermittelt.

Diese zeigten sehr grosse Genexpressionsunterschiede vor allem nach der *Xtg* Infektion. Genexpressionsunterschiede zwischen den *Xtg* infizierten Pflanzen und den kontrollbehandelten Pflanzen des resistenten Genotyps führte zur Identifizierung von einigen interessanten Kandidatengenen, die mit Bakterienwelke Resistenz assoziiert sein könnten. Unter anderen waren dies ein Gen, das für den low silicon transporter 1 (Lsi1) kodiert und eines, das für das germin-like protein 6 (GLP6) kodiert.

Um auch Gene von *Xtg* zu kennen, die für die Interaktion mit *L. multiflorum* von grosser Wichtigkeit sein könnten, wurde das *Xtg* Genom nach bekannten Virulenzgenen aus anderen phytopathogenen *Xanthomonas* spp. untersucht. Von allen bekannten Virulenzfaktoren von *Xanthomonas* stellt das Typ III Sekretionssystem (T3SS) den wichtigsten Virulenzfaktor dar. Das T3SS ist für die Übertragung von Effektor Proteinen in die Wirtszelle zuständig. Diese Effektoren können die Krankheitsentwicklung unterstützen und/oder Resistenzmechanismen des Wirts unterdrücken. Es wurden konservierte Primer auf Grund von Sequenzen publizierter *Xanthomonas* Genome entwickelt, um *Xtg* nach Genen zu untersuchen, die für T3SS Bestandteile kodieren könnten. Gene, die für Bestandteile des T3SS kodieren sind typischerweise hoch konserviert. Trotzdem wurde mit dieser Methode lediglich das *hrpG* Gen amplifiziert und sequenziert. Durch homologe Rekombination wurde eine Mutante generiert, die das *hrpG* Gen nicht mehr hat (Kapitel 4). Diese Mutante war signifikant weniger virulent bei der Infektion von *L. multiflorum* Pflanzen. *In planta* Analysen zeigten jedoch, dass die *ΔhrpG* Mutanten sich noch immer sehr stark vermehren konnten und nach 28 Tagen noch in grosser Zahl im Pflanzenmaterial vorhanden waren.

Weil phylogenetische Studien zeigten, dass *Xtg* nur wenig verwandt ist mit den übrigen sequenzierten *Xanthomonas* spp. und um weitere Gene des T3SS zu finden, wurde das gesamte Genom vom *Xtg*29 Isolat mit der 454 Methode sequenziert (Kapitel 5). Diese Methode ermöglichte eine vorläufige Analyse des T3SS Genclusters und eine Analyse von Genen, die für Effektoren und/oder andere virulenz-assoziierte Faktoren kodieren. Weitere T3SS Mutanten und Analysen von Effektor Proteinen werden in Zukunft die Identifizierung von Resistenzgenen unterstützen und zu einem noch besseren Verständnis dieser Wirt-Pathogen Interaktion führen.

Zusammenfassend stellen die Erkenntnisse aus dieser Studie einen elementaren Baustein dar, diese Interaktion in grossem Umfang zu verstehen. Es wurden sowohl Resistenzmechanismen der Pflanzen, als auch Virulenzmechanismen der Bakterien umfangreich untersucht und das erarbeitete Wissen stellt eine essentielle Grundlage dar für die Entwicklung von Marker gestützter Züchtung.

1 General introduction

1.1 Grasslands – important ecosystems

Grasslands are among the largest ecosystems in the world and cover approximately 70% of the global agricultural area and 26% of the global land area (FAOSTAT, 2008). Eighty per cent of the world's cow milk and 70% of the world's beef and veal are produced from temperate grasslands. Grasslands are also highly valued for preventing soil erosion and improving soil fertility. In addition, they protect water resources, are a promising resource to conserve biodiversity, and they also may enhance the landscape by recreational and aesthetic impacts (Humphreys, 2005). Since almost all European grasslands are more or less created, modified and/or maintained by agricultural activities, they are defined as 'semi-natural' although their plant communities are natural (Reidsma *et al.*, 2006). Semi-natural grasslands are usually species-rich, whereas modern managed grasslands are usually dominated by *Lolium*, *Festuca* and *Trifolium* species. However, depending on the climate and the intensity of use *Phleum*, *Dactylis*, and *Medicago* species are also commonly present (reviewed in Reheul *et al.*, 2010). Since grasslands are the main source of natural low-cost feed for ruminants, their importance for the future is increasing. The amount of meat consumed in developing countries has risen threefold faster compared to developed countries within the last decade. In general, nearly everywhere indigent people are eating more animal products as their incomes rise and as they become urbanized (Delgado, 2005). This expected increase in livestock production will represent fundamental challenges to agriculture and the environment worldwide.

1.1.1 The importance of ryegrasses in grassland production

With the growing demand for more meat and milk, the main role of grass species in temperate agriculture is to provide forage for ruminant animals. In grasslands, ryegrasses (*Lolium* spp.) are of major importance, as they represent together with a few fescue species, the most fundamental components of temperate grasslands worldwide providing the basis for grassland production systems (Wilkins & Humphreys, 2003). Italian ryegrass (*Lolium multiflorum* Lam.) and perennial ryegrass (*L. perenne* L.) both are considered to be the most favorable options for forage-livestock systems in natural and permanent grasslands, since they possess high yield potential and excellent forage quality features such as palatability, nutritive value and digestibility (Gilliland *et al.*, 2000). In temperate grasslands, ryegrasses are usually grown in mixtures with forage legumes such as red clover (*Trifolium pratens*e) and Lucerne (*Medicago sativa*). Both ryegrass species are used for intensive forage production, so they require an appropriate supply of nutrients to exhibit high productivity (Humphreys, 2005). Italian ryegrass is typically a cool-season annual bunchgrass native to southern Europe and it

may be used in many different environments, especially when fast cover is required. In addition to a high yield potential, it establishes well and is suitable for many different soil types ranging from dry to very moist and varying edaphic conditions ranging from pH 5.5 to pH 8. The close relative perennial ryegrass is especially persistent with a high tolerance to grazing, and therefore it represents an important component in pasture-livestock systems. Due to its strong seedling vigour and good tolerance to low mowing height, perennial ryegrass is also used extensively on golf course fairways, athletic fields and home lawns. Although Italian ryegrass has a higher yield potential than perennial ryegrass, it is rather grown for silage and hay production than on pastures. This is primarily due to low grazing tolerance and due to its poor ability to withstand summer and winter stresses. Therefore, breeding for improved traits such as resistance to abiotic and biotic stress is commonly applied in forage grasses.

Main goals in breeding ryegrasses include improved resistance to abiotic and biotic stress, and increased seed yield (reviewed in Humphreys *et al.*, 2010), increased dry matter (DM) yield and quality. Quality improvement especially refers to increased nutritional quality features such as digestibility and the proportion of water-soluble carbohydrates, proteins, and lipids. Improving resistance to abiotic stresses aims to increase tolerance to freezing temperatures, prolonged snow cover, low light intensities during winter, heat, drought, high light intensities (leading to oxidative damage), anoxia resulting from ice encasement, flooding and slurry application and high concentrations of salt, aluminium or heavy metals in the soil. The objective of improving resistance to biotic stress is to enhance resistance against diseases and pests. Diseases that limit ryegrass production include rusts (caused by *Puccinia* species), *Microdochium nivale* (the pathogen that causes pink snow mold), *Fusarium* patch, ergot (caused by *Claviceps purpurea*), *Drechslera* ssp., bacterial wilt (caused by *Xanthomonas translucens* pv. *graminis*). Italian ryegrass is particularly susceptible to bacterial wilt caused by *Xanthomonas translucens* pv. *graminis* (Suter *et al.*, 2005).

1.1.2 Systematics and genetics of the genus *Lolium*

The family of the *Poaceae* contains more than 700 genera and 10,000 species (Fig. 1.1; Gaut, 2002). Phylogenetically, the different species of the grass family separated in early Miocene. Although other angiosperm families contain more species and genera, the plant family of the *Poaceae* comprises numerous major cereal crop species such as rice (*Oryza sativa* L.), wheat (*Triticum* spp.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) and oat (*Avena sativa* L.). Despite differences based on genome-wide duplications and chromosome rearrangements, the genomes of the species of the *Poaceae* family display a high level of conserved synteny

(reviewed in Devos, 2005). Therefore, knowledge of comparative genomics for these species can be applied across species which has become a valuable tool for the identification of orthologous genes. The genus *Lolium* is closely related to the *Festuca* genus, which is one of the largest genera within the *Poeae* comprising more than 500 species (Fig. 1.1; Catalan *et al.*, 2004). Species of the genus *Lolium* are diploid with 7 pairs of chromosomes which form part of a *Lolium/Festuca* polyploid complex.

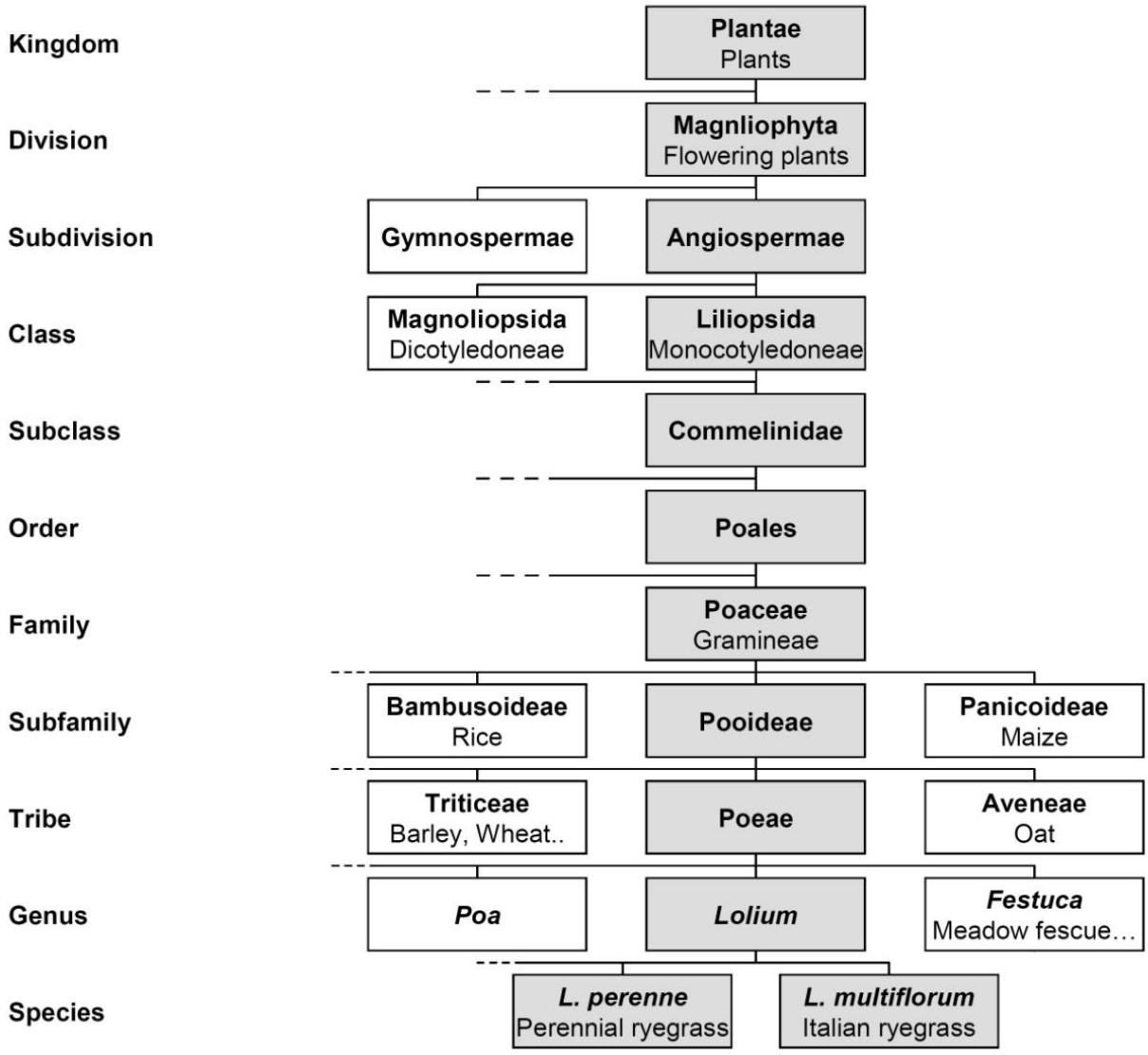

Figure 1.1 Taxonomy of the grass family (*Poaceae*) according to Gaut (2002). The figure was adopted from Studer (2008).

Many *Lolium* and *Festuca* species show some interfertility although two main groups can be distinguished based on pollination behavior and ease of hybridization. However, phylogenetic studies using ribosomal internal transcribed spacer (ITS) and chloroplast *trn*L-F sequences

included species of the genus *Lolium* in a '*Festuca*' clade together with a few *Festuca* spp. and *Mycropyropsis* spp. (Catalan *et al*., 2004). Since most diploid representatives of this clade are endemic to the Mediterranean, these species have been proposed to have undergone speciation in this region. Since the *Lolium* spp. clustered so closely to a number of *Festuca* spp. and close homologies between *L. multiflorum* and *Festuca arundinacea* and *F. pratensis* exist, the phylogeny of the genus *Lolium* is still highly controversial. The species of Italian ryegrass (*L. multiflorum*) is additionally divided into two different subspecies or variations called *L. multiflorum* ssp. *italicum* (Italian ryegrass) and *L. multiflorum* ssp. *multiflorum* (Westerwolths ryegrass). The former requires vernalization and may survive several years and the latter is an annual variation (reviewed in Humphreys *et al.*, 2010).

1.2 *Xanthomonas* spp. – destructive plant pathogens

Bacteria belonging to the genus *Xanthomonas* comprise some of the economically most destructive plant pathogens potentially causing severe losses on various crops all over the world (Agrios, 2005; Swings & Civerolo, 1993). *Xanthomonas* spp. belong to the subdivision of gamma proteobacteria, are Gram-negative, rod shaped, obligate aerobes, and have a single polar flagellum, which renders them motile. Their optimal growth temperature is between 25 and 30˚C, and colonies are typically yellow due to the presence of the membrane-bound pigment xanthomonadin, which is thought to protect the bacteria from photobiological damage (Rajagopal *et al.*, 1997; Starr & Stephens, 1964). The host range of *Xanthomonas* spp. covers approximately 124 monocotyledonous and 268 dicotyledonous plants belonging to 68 plant families and 240 genera (Hayward, 1993) including a number of forage grass species belonging to the genera *Agrostis, Alopecurus, Bromus, Dactylis, Festuca, Lolium, Phleum, Poa* and *Trisetum* (Leyns, 1993).

1.2.1 Diseases caused by *Xanthomonas* spp.

Some *Xanthomonas* species and pathovars are of great economic importance, especially in regions with warm climate (Leyns *et al.*, 1984) where they infect crops such as rice (*Oryza sativa* L.), beans (*Phaseolus* spp. L.), cassava (*Manihot esculenta* C.), cotton (*Gossypium* spp. L.), tomatos (*Solanum lycopersicum* L.) and citrus (*Citrus* spp. L.). The bacteria presumably persist as epiphytes on plant surfaces before they either enter the plant via natural openings such as stomata and hydathodes, or via induced breaches such as wounds. Once inside the plant tissue, many *Xanthomonas* spp. exhibit tissue-specificity, invading either the host xylem vessels (vascular pathogen) or the interveinal mesophyll apoplast (mesophyllic pathogen). Typical symptoms include either necrosis, gummosis or vascular and parenchymatous

diseases on leaves, stems or fruits, leading to considerable losses in food and feed production (Fig 1.2).

Figure 1.2 Examples of diseases caused by *Xanthomonas* spp. A) Citrus canker on leaves and fruit caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*: source: http://www.invasive.org/images/). B) Bacterial spot disease on tomato caused by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv;* source: http://plaza.ufl.edu/jbjones/joneslab/). C) Bacterial leaf streak disease on wheat leaves caused by *Xanthomonas translucens* pv. *translucens* (*Xtt*; source: http://photos.eppo.org/).

Chemical control against diseases caused by *Xanthomonas* spp., using antibiotics is not desired in food and feed production due to the emergence of antibiotic resistant bacteria and/or horizontal gene transfer of these antibiotic resistance genes among bacteria. Therefore, breeding for resistant cultivars is currently the only applicable means to control the disease and reduce excessive yield losses (Yang *et al.*, 2003).

1.2.2 Systematics of *Xanthomonas*

Originally, members of the genus *Xanthomonas* were all grouped into separate species on the basis of their host range. However, this led to an unreasonably large number of nomenspecies, which later resulted in a reclassification according to the classical nomenclature. The consequence was a large-scale partial merge into the single species *Xanthomonas campestris* which was then subgrouped into different pathovars (Dye & Lelliott, 1974). Later, another reclassification was proposed by Young *et al*. (1978) based on the former taxonomy system. However, investigations based on DNA hybridization (Vauterin *et al.*, 1995), 16S ribosomal RNA gene sequencing (Hauben *et al.*, 1997), DNA fingerprinting (Rademaker *et al.*, 2000), and sequencing of the gyraseB gene (Parkinson *et al.*, 2009) have revealed that the current classification is not a reflection of genetic relationships. Currently, there is a still an ongoing debate concerning approximately 19 species and more than 140 pathovars (Rademaker *et al.*, 2005; Schaad *et al.*, 2000; Vauterin *et al.*, 2000). In this thesis, the taxonomy proposed by Vauterin *et al.*, (1995) is used (Fig 1.3).

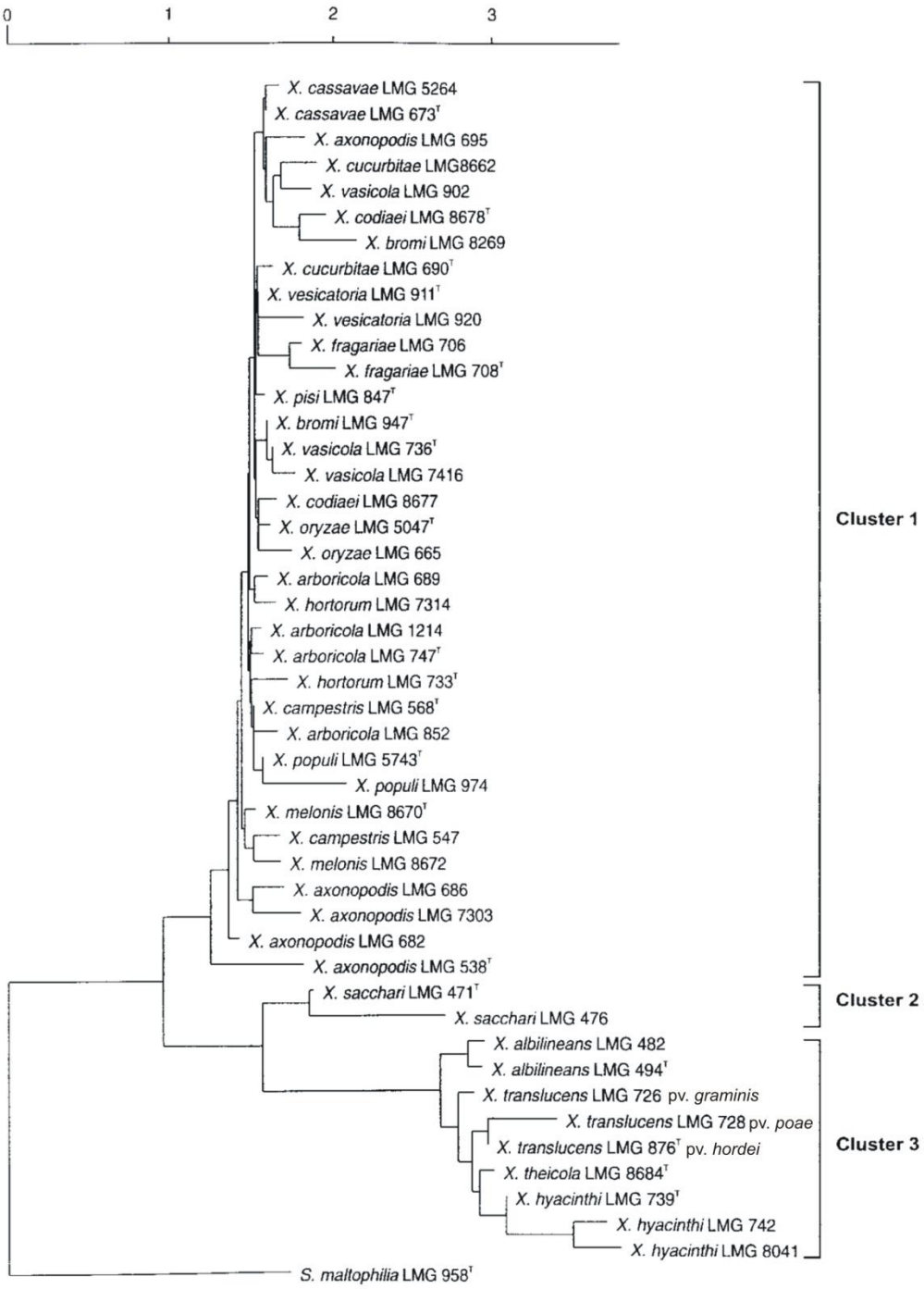

Figure 1.3 Neighbor-joining dendrogram of estimated phylogenetic relationships based on pairwise comparisons of partial 16S rDNA sequences of a number of *Xanthomonas* strains (Hauben *et al.*, 1997).

The species of *Xanthomonas translucens* consists of at least ten different pathovars and is exclusively pathogenic to members of the grass family such as wheat, barley, rye and forage grasses. According to the phylogeny presented above, *X. translucens* clusters together with *X. theicola, X. hyacinthi and X. albilineans* into cluster 3 (Fig. 1.3).

1.2.2 *Xanthomonas translucens* pv. *graminis* causing bacterial wilt of forage grass

Bacterial wilt caused by *Xanthomonas translucens* pv *graminis* (*Xtg*) is a serious disease in forage grasses such as *Agrostis*, *Alopecurus*, *Bromus*, *Dactylis*, *Festuca*, *Lolium*, *Phleum*, *Poa* spp. and *Trisetum* spp. (Paul & Smith, 1989), and may lead to complete yield losses in pure and mixed stands depending on cultivar susceptibility. On average, it is estimated to account for annual forage yield losses of 10–15% in cultivated grassland (Suter *et al.*, 2005). The disease was first observed in the early seventies and the causal agent was initially named *X. graminis* (Egli *et al.*, 1975). Four different pathovars were identified to infect forage grasses (Egli & Schmidt, 1982). These four pathovars were classified as *Xanthomonas campestris* pathovars (pvs.) and were named according to the species of their host range (pv. *phlei*, pv. *arrhenateri*, pv. *poae*, and pv. *graminis*). While the first three pathovars are restricted to one genus, *X. t.* pv. *graminis* can infect a number of different forage grass species and has the widest host range. More recently, these four pathovars of *Xanthomonas* were renamed to *Xanthomonas translucens* pvs (Vauterin *et al.*, 1995). Of the four pathovars, *Xtg* is the most abundant and has a wide-spread geographical distribution being prevalent in most pastures and meadows of Europe, the USA and Australasia (Leyns, 1993). Infection of the host plants occurs primarily through wounded tissue. Consequently, disease dispersal is promoted by mowing with contaminated equipment during hay and silage production. Typically, disease dispersal through mowing is more severe when the meadows are wet. Therefore, it is recommended to wait for dry conditions until mowing the meadows (Schmidt, 1988a). Beside moisture at harvest, cutting height affects disease development such that a lower mowing height results in faster and more severe disease symptom development (Leyns *et al.*, 1988). Although living bacteria of *Xtg* have been isolated from the glumes of grass seed, neither seed-transmission (Schmidt, 1989) nor soil transmission have been demonstrated. Some previous studies have claimed occurrence of *Xtg* entering through stomata but experimental evidence has not yet been provided (Wang & Sletten, 1995).

Typical disease symptoms such as wilting of the leaves and tillers occur approximately between one or two weeks after infection depending on environmental conditions. In severe cases, the plant may die within only a few days after infection. More rapid symptom development usually occurs under warm and dry conditions. This is explained by that fact that optimal growth temperatures of *Xtg* are at 28˚C. The physiological state of the plant also seems to be important for disease severity. Most severe symptoms are observed during heading and flowering. In addition, newly sown plots are also more affected than well established meadows (Schmidt, 1988a). Wilting and necrosis of the infected leaves and tillers

begins at the infection site or at the tip of the leaves and progresses towards the base of the plant (Fig. 1.4).

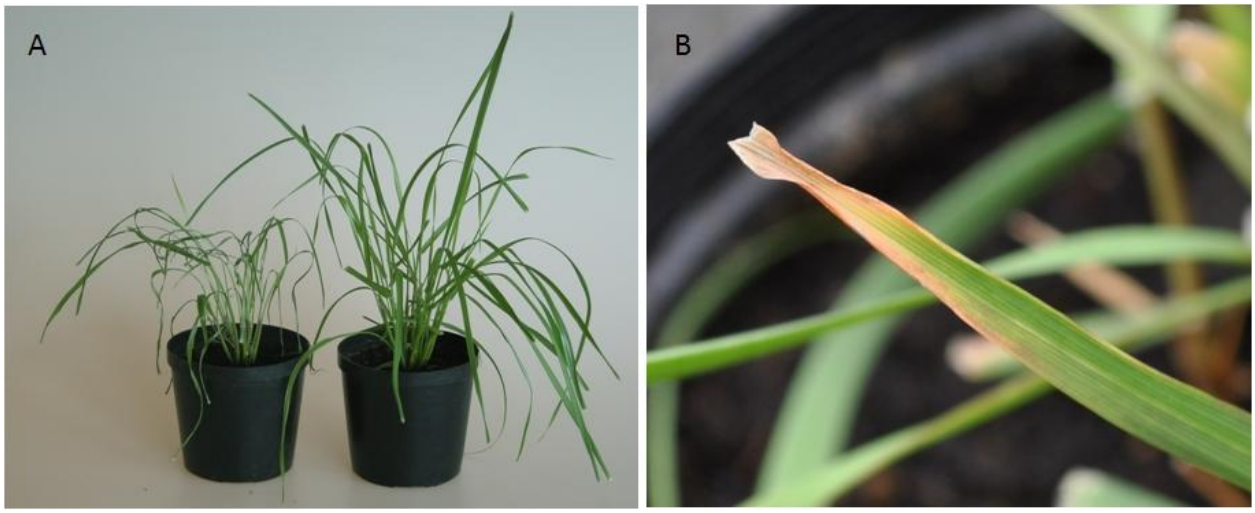

Figure 1.4 Symptoms of *Xanthomonas translucens* pv. *graminis* (*Xtg*) infection on *L. multiflorum* plants. A) Comparison of two different plants of a susceptible genotype infected with *Xtg* and exposing disease symptoms (left), and an non-infected healthy plant of the same genotype (right). B) Close up image of a plant with early disease symptoms with leaf tissue necrosis at the infection site progressing down to the base of the plant.

The pathogen spreads within the xylem vessels, and therefore yellow stripes along the vascular tissue can also be observed on adult plants. Depending on disease severity, *L. multiflorum* plants can recover from bacterial wilt disease after cutting the plants back and removing affected tillers. Only very moderate symptoms are then observed until 21 days after cutting (see chapter 2). The symptoms of all four pathovars causing bacterial wilt on forage grasses are similar to each other.

Several strategies to limit *Xtg* infection have been elaborated. These included the induction of systemic acquired resistance with phylloplane, non-pathogenic bacteria (Schmidt, 1988c) and the disinfection of contaminated mowing equipment. However, neither strategy has provided efficient tools to prevent bacterial wilt infection (Schmidt, 1988b). Therefore, breeding for resistance to *Xtg* based on artificial seedling inoculation is implemented in the breeding schemes of ryegrasses (Humphreys *et al.*, 2010) and this approach represents the only applicable means to prevent excessive yield losses in forage production. Efficiency of breeding for resistance to *Xtg* is demonstrated with the cultivar Axis bred at Agroscope Reckenholz-Tänikon when compared to the moderately susceptible cultivar Ligrande or the highly susceptible cultivar Adret. The Swiss cultivar Axis is clearly more resistant and had undergone two cycles of screening for *Xtg* resistance, whereas the other cultivars had not been selected for *Xtg* resistance (Kölliker *et al.*, 2006).

1.3 Virulence factors from *Xanthomonas* spp.

The establishment of infection by phytopathogenic bacteria is mediated by virulence factors. Virulence factors can generally be defined as all bacterial products or strategies that contribute to the ability of the bacterium to cause disease. Current research in molecular plant pathology has been primarily focused on the identification of bacterial virulence factors that contribute to the plant–pathogen interaction. In order to successfully colonize host plants, phytopathogens must be able to adhere to the plant surface and invade the mesophyll or the vascular system to acquire nutrients and resist plant defence responses. Bacteria are often also dependent on different kinds of secretion systems that secrete proteins and/or DNA into the extracellular milieu or directly into the host cell (Fig. 1.5).

Depending on the secreted molecule, pathogens can make use of a combination of different protein secretion systems in order to multiply inside their host. For *Xanthomonas* spp., the most extensively studied secretion system is the type III secretion system (T3SS). The T3SS is responsible for the secretion of various effectors into the host cell. Resistance mediated by *R*-genes of the host is predominantly based on the specific recognition of type III secreted bacterial effectors (see 1.4.1). The result of specific recognition is the hypersensitive response which inhibits pathogen reproduction. When not recognized, these effectors may suppress host defence mechanisms and promote virulence processes of the pathogen. Therefore, characterization of the genes encoding effector proteins may enable the identification of resistance mechanisms inside the host plants. In the following, the different secretion systems and other virulence factors present in *Xanthomonas* spp. and also potentially important for *Xtg* infection will be described.

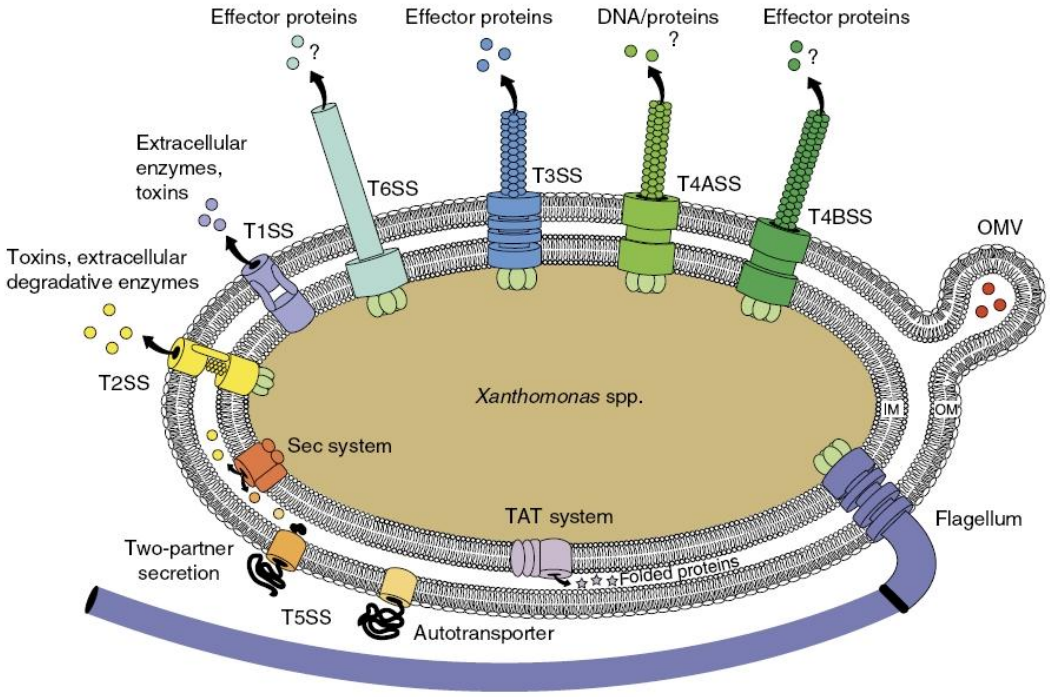

Figure 1.5 Schematic representation of protein secretion systems from *Xanthomonas* spp. (Büttner & Bonas, 2009). Six types of protein secretion systems are encoded. The T2SS (yellow) and T5SS (orange) depend on the general secretory pathway (red: Sec) or the twin-arginine translocation (light purple: TAT) system (reviewed in Palmer *et al.*, 2005) for protein transport across the inner membrane. The T3SS (blue), T4SS (light green) and T6SS (light blue) are associated with extracellular pilus structures and presumably translocate proteins into the host cell. So far, protein translocation has experimentally been proven only for T3SS. Only in a few cases does protein secretion depend on the formation of outer membrane vesicles (OMV). IM, inner membrane; OM, outer membrane; TAT, twin-arginine translocation.

1.3.1 The type III secretion system (T3SS)

The type III secretion system (T3SS) is an important virulence factor of major Gram-negative phytopathogenic bacteria (Galan & Collmer, 1999). It is responsible for the secretion of effectors in most *Xanthomonas* spp., *Pseudomonas syringae*, *Erwinia* spp. and *Ralstonia solanacearum*. It is expected that *Xtg* also makes use of a T3SS in order to colonize forage grasses. The genes encoding the T3SS of phytopathogenic bacteria are typically localized in large gene clusters on either the chromosome or a plasmid (Arnold *et al.*, 2003) and are termed *hrp* (hypersensitive response and pathogenicity) genes. Eleven genes are highly conserved in plant and animal pathogenic bacteria and were therefore termed *hrc* (*hrp* conserved Bogdanove *et al.*, 1996). In addition, *hpa* (*hrp* associated) genes have been identified which contribute to pathogenicity but are not essential (Huguet *et al.*, 1998). Expression of the *hrp* gene cluster results in the formation of a membrane-spanning secretion

apparatus called the Hrp-pilus or type III injectisome, which mediates the delivery of bacterial effectors into the host cell (Fig. 1.6; Cornelis & Van Gijsegem, 2000).

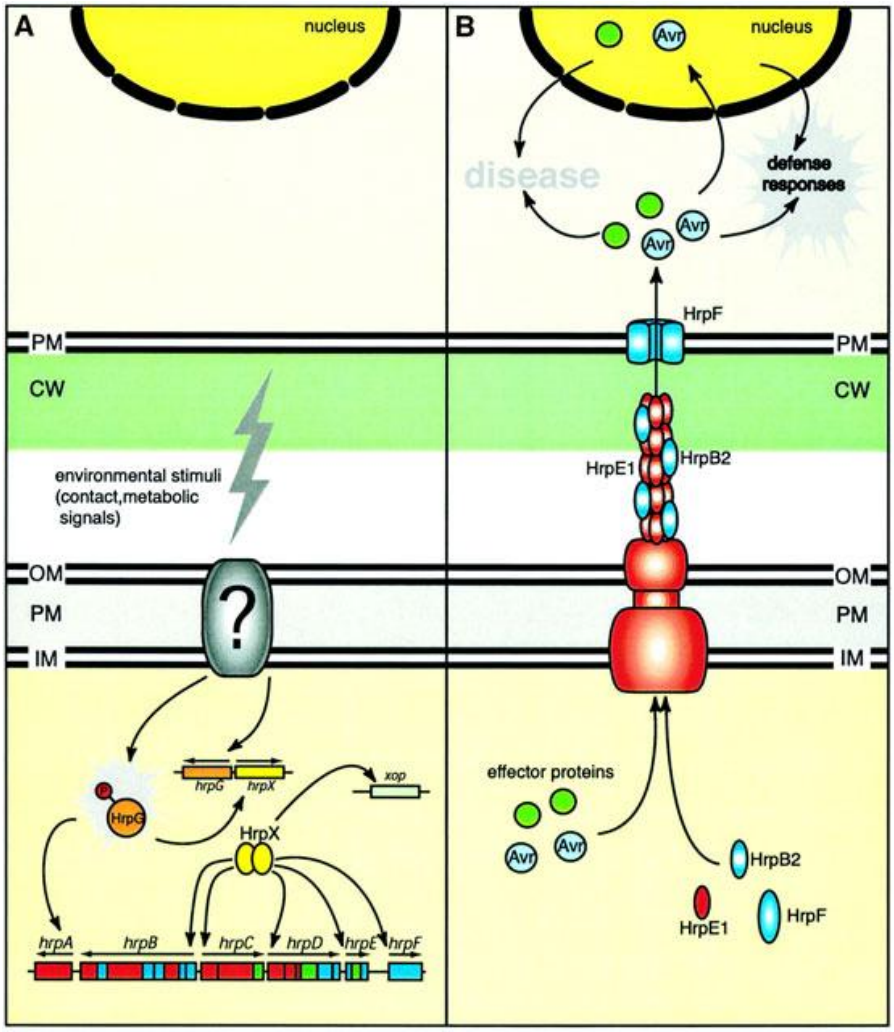

Figure 1.6. Model for *hrp* gene regulation and type III secretion in *X. campestris* pv. *vesicatoria* (*Xcv*) (Büttner, 2002). A) A signal transduction system in the bacterial envelope (so far unknown) senses environmental stimuli and transduces the signal to HrpG, which activates the expression of *hrpA*, *hrpB-hrpF* and *xop* via HrpX. B) Expression of *hrp* genes is essential for the T3SS which mediates the secretion of Hrp proteins required for T3SS assembly and translocation of effector proteins.

The components of the T3SS apparatus share significant similarities with components of the flagellum such as export of hook and filament components (reviewed in Macnab, 2003). However, there are phylogenetic studies that do not support the hypothesis that the T3SS was derived from the flagellum but rather indicate that the T3SS is equally as ancient as the flagellum and both share a common ancestor (Gophna *et al.*, 2003). The T3SS apparatuses from Gram-negative bacteria have evolved and have been grouped into seven different families based on phylogenetic analyses (reviewed in Cornelis, 2006). Of the seven families, the T3SS injectisomes of phytopathogenic bacteria are divided into two different families

called Hrp1 and Hrp2. The injectisomes of the genus *Xanthomonas* (γ-proteobacteria) belongs to the Hrp2 family together with *Burkolderia* (β-proteobacteria) and *Ralstonia* (β -proteobacteria). As this example shows, phylogenetic analyses of the T3SS have revealed that the evolutionary trees of the bacteria themselves compared to the trees of genes encoding their T3SS do not resemble each other. The genes encoding the T3SS components are therefore proposed to have been distributed horizontally in late bacterial evolution.

In contrast to the conserved T3SS apparatus, the genes encoding the effectors of *Xanthomonas* spp. are very diverse. To date, more than 100 different effector proteins that can be divided into 39 different so-called Xop (*Xanthomonas* outer protein) groups based on sequence similarity (White *et al.*, 2009) are known that are secreted via the T3SS. Depending on the strain, approximately 20–30 effectors with overlapping activities and diverging composition are typically secreted by one single *Xanthomonas* strain (reviewed in Büttner & He, 2009; Büttner & Bonas, 2010). These effectors can fulfil multiple functions, such as interference with host immunity or they may facilitate nutritional and virulence processes of the pathogen (Büttner & He, 2009). At the same time, they represent essential determinants of pathogenicity on susceptible plants and are required for the induction of the hypersensitive response (HR) on resistant plants (White *et al*., 2000). Some effectors such as the transcription activator-like effectors (TALEs) are structurally and functionally well-characterized, and have been the subject of more than 20 years of ongoing research in the pepper and tomato pathogen *Xanthomonas campestris* pv. *vesicatoria* (reviewed in Bogdanove *et al.*, 2010). TALEs (also called AvrBs3/PthA-family effectors) and closely related proteins have been found in several but not all phytopathogenic *Xanthomonas* species and *Ralstonia solanacearum* (Mukaihara *et al.*, 2010). TALEs consist of a common N-terminus required for type III secretion and a C-terminus containing a nuclear localization signal (NLS) and an acidic activation domain (AAD). Nearly all characterized and cloned *R*-genes that are effective against *Xanthomonas* spp. rely on detection of or interaction with TALEs.

1.3.2 Other secretion systems

Although poorly understood, *Xanthomonas* spp. may also make use of secretion systems other than the T3SS, that potentially also play important roles in the interaction with their host. In *Xanthomonas* spp. genes for all known protein transport systems of Gram-negative bacteria have been identified, i.e. the Sec, signal recognition particle, and TAT pathways; type I, type II, and type III, type IV secretion systems of different types, type V autotransporters, two-partner secretion systems, and a type VI secretion system. For example, the T2SS typically

consisting of 12–15 components, is associated with the bacterial inner membrane (reviewed in Sandkvist, 2001). It is responsible for the secretion of toxins and extracellular enzymes such as proteases, lipases and cell wall-degrading enzymes. These may act as virulence factors facilitating host invasion by destruction of tissues. Type II secretion involves two steps in which proteins containing an N-terminal signal peptide are first translocated across the cytoplasmic membrane via the sec machinery. Then, following removal of their signal peptides and release into the periplasm, the mature proteins cross the outer membrane in a separate step. Expression of the genes for these proteins are controlled by quorum-sensing mechanisms or are strictly regulated by the environment at the site of colonization. Two different variants of the T4SS, which has been well characterized in the phytopathogen *Agrobacterium tumefaciens*, have also been found in *X. campestris* pv. *vesicatoria* (*Xcv*; Thieme *et al.*, 2005).

1.3.3 Extracellular polysaccharides (EPS)

Almost all *Xanthomonas* spp. (except for *Xanthomonas albilineans*) produce a characteristic extracellular polysaccharide (EPS) called xanthan. Xanthan is a heteropolysaccharide consisting of repeating pentasaccharide units with a cellulose-like backbone and trisaccharide side chains of two mannose and one glucuronate residues. Xanthan makes the bacterial colonies appear mucoid (Fig. 1.7 A) and it is commercially produced by fermentation for thickening and emulsifying nutritional and pharmaceutical products (Becker *et al.*, 1998). The production of xanthan in the bacteria is directed by several genes located on the *gum* gene cluster. The *gum* gene cluster typically consists of 12 genes (*gumB* to *gumM*) which are highly conserved among *Xanthomonas* spp (Katzen *et al.*, 1998). Due to the highly hydrated and anionic consistency of xanthan, it is expected that it protects bacteria from environmental stresses such as dehydration and toxic compounds. In addition to protection, for vascular pathogens, xanthan might be responsible for wilting of host plants by blocking the water traffic in xylem vessels (Fig. 1.7 B; Chan & Goodwin, 1999). Although inconsistencies in observations and data connected to xanthan production and its function related to pathogenicity exist, expression of the *gum* gene cluster most likely contributes to epiphytic survival and is not required for pathogenicity (Dharmapuri & Sonti, 1999; Dunger *et al.*, 2007; Katzen *et al.*, 1998; Kemp *et al.*, 2004; Rigano *et al.*, 2007).

However, xanthan has also been suggested to suppress basal plant defense responses such as callose deposition in the plant cell wall, which presumably occurs by chelation of divalent calcium ions in the plant apoplast and are required for the activation of plant defense responses (Aslam *et al.*, 2008). Further, xanthan has been shown to be involved in the

formation of bacterial biofilms in *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* (Dow *et al.*, 2003; Rigano *et al.*, 2007; Torres *et al.*, 2007) in which bacteria attach to each other forming an extracellular polymeric matrix consisting of proteins, lipids and EPS (Sutherland, 2001). Biofilm formation might contribute to bacterial epiphytic survival before colonization of the plant intercellular space because it presumably provides protection against antibiotics and host defense responses prior to attachment of vascular bacteria to xylem vessels (Stoodley *et al.*, 2002).

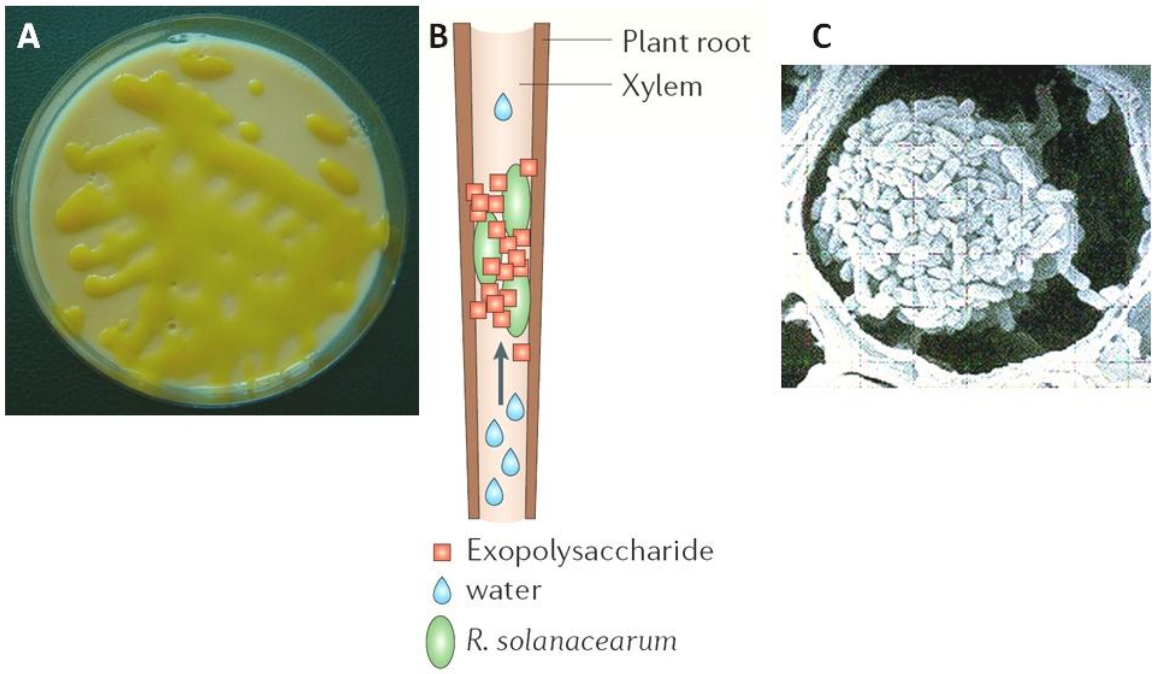

Figure 1.7. Exopolysaccharides (EPS) and their importance for bacterial wilt symptoms. A) Agar plate containing glucose, yeast extract and calcium carbonate (GYC) media overgrown with *Xanthomonas translucens* pv. *graminis* colonies. B) Model for bacterial wilt symptoms caused by *Ralstonia solanacearum* (Abramovitch *et al.*, 2006). After adherence to the xylem vessels, the bacteria produce EPS which results in blocking of water traffic and the typical bacterial wilt symptoms. C) Scanning electron microscope image of a xylem vessel in a forage grass filled with *Xanthomonas translucens* pv. *graminis* bacteria (source: http://ipm.illinois.edu/diseases).

1.3.4 Lipopolysaccharides (LPS)

In addition to EPS, lipopolysaccharides (LPS) represent another group of surface-associated virulence factors of *Xanthomona*s spp. and other Gram-negative phytopathogens. Similar to EPS, LPS are essential components of the bacterial outer membrane and may protect the bacteria in harsh environments. LPS is a tripartite molecule consisting of a membrane-anchored lipid A, a core oligosaccharide and polysaccharide side chains (O-antigen; reviewed

in Raetz & Whitfield, 2002). In *X. campestris* pv. *campestris*, the synthesis of LPS is directed by the *wxc* gene cluster, which comprises 15 different genes (Vorhölter *et al.*, 2001). Mutations in the *wxc* gene cluster causes higher sensitivity in unfavorable conditions for *Xanthomonas* spp. and might therefore lead to an attenuation of bacterial virulence as it has been shown for *X. campestris* pv. *campestris* and *X. campestris* pv. *citrumelo* (Dow *et al.*, 1995; Kingsley *et al.*, 1993; Newman *et al.*, 2001). Comparative sequence analysis of *wxc* gene clusters and whole genome sequences have revealed that LPS gene clusters of different *Xanthomonas* spp. are highly variable in number and identity of genes. Therefore, LPS genes presumably are subject to a strong diversifying selection driven by different species, pathovars or even strains (Lu *et al.*, 2008). Variations in LPS composition facilitate bacteria to avoid recognition of resistance mechanisms of the plant and presumably also affect bacterial resistance to phage adsorption and/or infection (Ojanen et al., 1993; Hung et al., 2002).

1.4 Concepts of disease resistance in plants

Similar to the variety of virulence factors that act specifically (effectors) or more general (EPS and LPS) to establish host colonization, there are several strategies that have evolved in plants that either enable evasion of, or resistance to, virulence factors of pathogens. One strategy involves morphological characters that enable plants to avoid infection. These include hairs or waxy surfaces that cover the epidermis and repel water needed by the pathogen to move along the surface before entering the host. Plants may also produce a wide variety of antimicrobial or chemical compounds such as glucanases, chitinases, phytoalexins, tannins, phenolic compounds, saponins, proteases or hydrolytic enzymes that act as general defense against invading pathogens. Once a pathogen has successfully invaded a plant, different resistance mechanisms mediated by resistance genes (*R*-genes) can counteract pathogen proliferation. Breeding for resistant cultivars is widely used for disease control and once identified, introgression of different *R*-genes in one cultivar is a promising option to obtain durable and effective resistance. In general, there are three different aspects of disease resistance that are considered when breeding for disease resistance: (1) inheritance of the resistance (qualitative/monogenic or quantitative/polygenic), (2) effectiveness (complete or partial), and (3) specificity (race-specific or general in its effectiveness). There has been a tendency to reduce these six characteristics into just two i.e. qualitative, race-specific and complete resistance versus quantitative resistance that is partially effective against all races. Despite the fact that only these two main concepts are explained below, it is important to keep

in mind that this is an oversimplification and different combinations can exist (reviewed in Stuthman *et al.*, 2007).

1.4.1 Qualitative (race-specific) resistance

In plant–pathogen interactions, qualitative (race-specific) resistance requires a dominant or semidominant resistance gene (*R*-gene) in the plant, and a corresponding avirulence (Avr) gene in the pathogen acting in a gene-for-gene manner (Flor, 1971). Recognition of the bacterial Avr products and the existence and expression of the corresponding host resistance gene (*R*-gene) products result in a incompatible interaction and the host is called resistant (reviewed in Baker *et al.*, 1997; Hammond-Kosack & Jones, 1997; Jones & Dangl, 2006). Incompatibility is often apparent because of a hypersensitive response (HR) characterized by a rapid local programmed cell death at the infection site within 24 hours (Scheideler *et al.*, 2002). The HR therefore enables the limitation of initial inoculum pressure or inhibits pathogen reproduction (Fig. 1.8).

When bacterial Avr products (effectors) are not recognized or the plant does not express the corresponding *R*-gene, the interaction is compatible and the host is susceptible. On the other hand, during the susceptible (compatible) interaction, water soaked lesions usually occur with a delay compared to the HR.

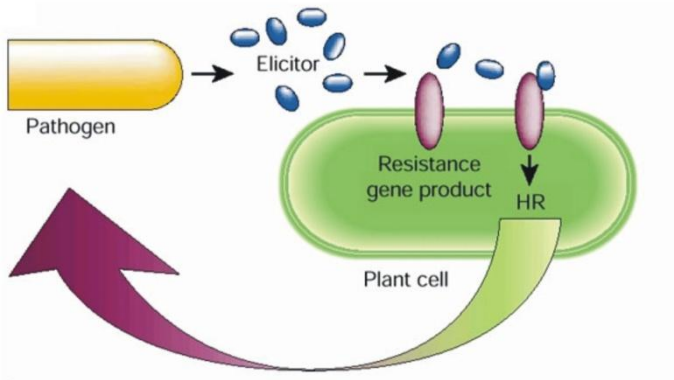

Figure 1.8 The hypersensitive response (HR) is triggered by the highly specific recognition of effectors (elicitors, blue) produced by pathogens (yellow) by a plant resistance gene (*R*-gene) product (purple). The HR inhibits pathogen reproduction (purple and green arrow; Stuiver & Custers, 2001).

Incompatibility of host-*Xanthomonas* interactions controlled both by the host *R*-genes and the bacterial Avr product has been demonstrated for a number of different crop species (e.g. rice, citrus, beans and tomato). For example, in rice where *X. oryzae* pv. *oryzae* (*Xoo*) causes bacterial blight, at least 33 race-specific genes conferring resistance to *Xoo* have been identified and seven have been cloned (Niño-Liu *et al.*, 2006; White & Yang, 2009).

Although qualitative resistance is highly effective for the control of many diseases, breeding for qualitative resistance can also lead to rapid break-down of resistance. This may occur because of accumulation of specific *R*-genes in host plant populations and cultivars resulting in an increased selection pressure and fast bacterial evolution. Races typically develop by single gene mutation and/or loss of specific *avr* genes (Brunings & Gabriel, 2003; Hua *et al.*, 2007; Leach *et al.*, 2001; Wydra *et al.*, 2004). This risk seems to be particularly high in major crops such as rice and wheat where cultivars are bred in lines and often consist of single genotypes all carrying these same specific *R*-genes. Thus, many corresponding avirulent bacterial isolates have evolved for the pathogens of these species (reviewed in: Salzberg *et al.*, 2008; Yang *et al.*, 2003).

1.4.2 Quantitative disease resistance

Quantitative resistance is more frequently observed, and in contrast to qualitative disease resistance it is controlled by various genes (polygenic) that provide cumulative effects contributing to disease resistance. As a consequence, disease resistance usually presents a continuous quantitative variation depending on environmental conditions. Overall, quantitative resistance is considered to be more durable when compared to race-specific, qualitative resistance, because less selection pressure on single virulence genes result in a slower pathogen evolution (reviewed in McDonald & Linde, 2002). In most cases, quantitative resistance is not complete and depends on a wide range of different mechanisms that render the plant more resistant. These mechanisms may include callose deposition, the development of physical barriers (lignin or waxy leaf surfaces), or the accumulation of antimicrobial compounds (phytoalexins, glucanases and chitinases). Although apparently more durable, quantitative resistance carries the disadvantage of providing only partial resistance and being less effective when conditions are particularly favorable for the pathogen (Geiger & Heun, 1989). Therefore, in order to obtain durable resistance, breeding aims at the application and management of durable single major *R*-genes, pyramiding of several *R*-genes or a combination of qualitative and quantitative resistance in one cultivar.

1.5 Improving resistance to bacterial wilt

1.5.1 Breeding for resistant forage grasses with recurrent selection

Recurrent selection of desired phenotypic traits for numerous generations and subsequent random mating of plants originating from natural genetic variation of cultivars and ecotypes is the most commonly applied breeding approach for forage species (Burton, 1992). Breeding

for resistant *L. multiflorum* cultivars is currently the only practicable means to control excessive yield losses caused by bacterial wilt. Therefore, resistance screening is a major objective in the breeding process of *L. multiflorum* cultivars (reviewed in Humphreys *et al.*, 2010). Improving *Xtg* resistance in the breeding schemes of ryegrasses is based on two cycles of artificial seedling inoculation and subsequent phenotypic selection. As it is the case for most forage grasses, *L. multiflorum* cultivars are bred in population-based breeding schemes leading to cultivars consisting of individual genotypes representing high levels of genetic diversity (Brummer, 1999; Kölliker *et al.*, 1999; Van Treuren *et al.*, 2005). Although resistance breeding has resulted in the development of cultivars with increased resistance to bacterial wilt, further breeding progress is difficult to obtain. This is partially due to allelic variation in the populations rendering resistance alleles difficult to control. Therefore, it is difficult to obtain cultivars with 'fixed' *Xtg* resistance and highly susceptible individuals may still occur in advanced breeding germplasm (Michel, 2001). In addition, complete resistance has not been achieved, and the population-based breeding schemes and the out-breeding pollination require a more elaborate breeding process for forage grasses compared to self-pollinating crops such as rice and wheat.

Marker-assisted selection (MAS) represents a promising option to complement and enhance phenotypic selection and 'fixing' resistance alleles. However, in order to use MAS in *L. multiflorum* breeding, it is necessary to understand in more detail the inheritance of resistance (qualitative or quantitative resistance) and identify the most important virulence factors that contribute to the plant–pathogen interaction. There are several tools employed by plant breeders to understand the genetics and concepts of disease resistance which are explained in the following.

1.5.2 Genetic linkage maps and quantitative trait loci (QTL)

Linkage mapping is a genetic tool for the identification of the position of genes or genetic markers relative to each other in terms of recombination frequency during meiosis (Tanksley, 1993). This permits the construction of linkage maps composed of genetic markers for a specific population. In contrast to physical maps, linkage maps do not represent specific physical distances along each chromosome. Usually, construction of a linkage map requires a population derived from sexual reproduction.

Linkage maps of forage grasses such as *Lolium* spp., were for a long time not as developed as those of major crop species such as cereals. However, the application of DNA markers such as restriction fragment length polymorphisms (RFLP), simple sequence repeats (SSR), amplified fragment length polymorphisms (AFLP), single nucleotide polymorphisms

(SNP) and Dart (Kopecky *et al.*, 2009) has resulted in numerous high density genetic linkage maps for forage grasses and *Lolium* spp. (Armstead *et al.*, 2004; Gill *et al.*, 2006; Hirata *et al.*, 2006; Jones *et al.*, 2002). Recently, some great effort has also been invested to combine the genetic linkage maps, developed for different mapping populations of *Lolium* spp., resulting in a combined consensus linkage map (Studer *et al.*, 2010). In the future, this map may serve as the reference map for genetic linkage studies in *Lolium* spp. (Fig. 1.9).

Based on such linkage maps, many important traits such as yield, quality or disease resistance are analyzed in order to understand the genetic basis of these complex traits (Asins, 2002). Quantitative trait loci (QTL) analysis consists of the joint analyses of genotype marker segregation and phenotypic values of individuals within a mapping population. Combined, this approach provides information about the chromosomal location, the number and the individual contribution of genetic loci involved in the control of a target trait. Thus, a QTL is a polymorphic locus with alleles that differentially affect the characteristic of a continuously distributed phenotypic trait.

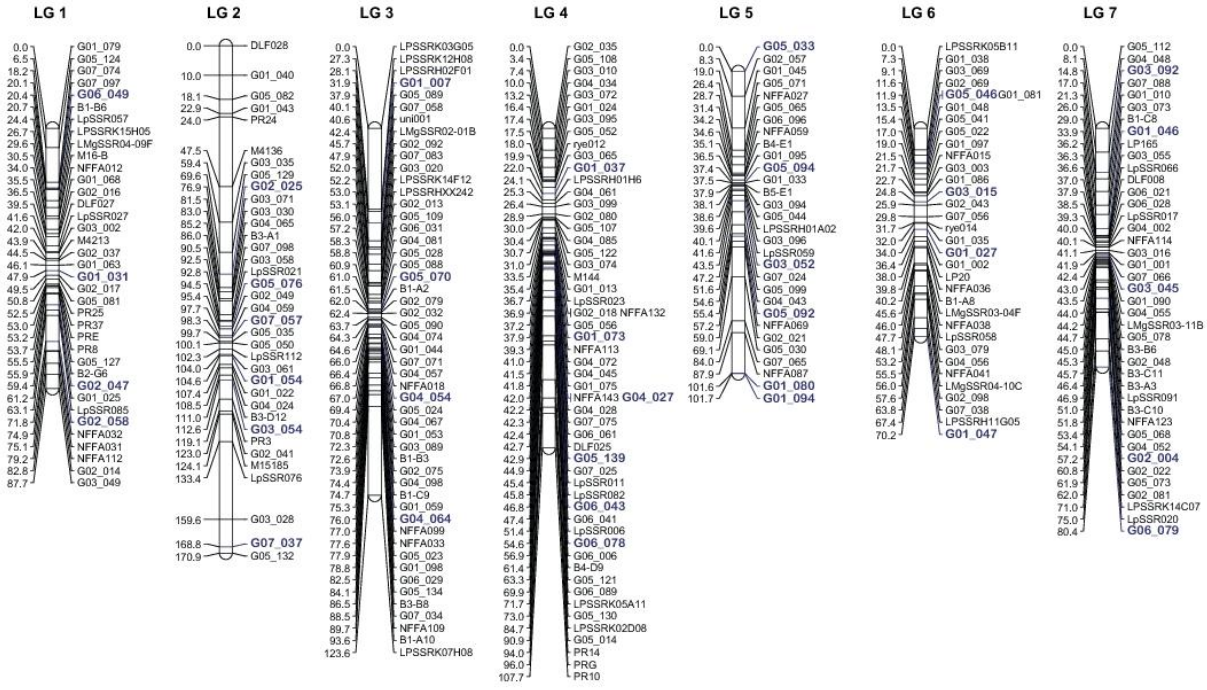

Figure 1.9 The consensus linkage map of *Lolium* spp. developed by Studer *et al.*, (2010). The consensus linkage map contains 284 simple sequence repeat (SSR) markers and is 742 centiMorgan (cM) long. The seven linkage groups (LGs) indicate the chromosomes and scale units are given in centiMorgan (cM).

1.5.3 Transcriptome analyses of host–bacterial pathogen interactions

In contrast to genetic analyses based on linkage mapping and QTL, transcriptome analyses are widely used to study the abundance of transcripts of a specific gene. This enables the determination of gene functions and pathways in different physiological and developmental processes. These include, for example, response to a specific stress such as pathogen infection, environmental conditions, or changes during a developmental process such as senescence or vernalization. A variety of methodologies for gene expression analyses are available and new ones are emerging with the description of whole genome sequences and the availability of novel high-throughput sequencing technologies. Since the establishment of RNA gel-blots, which are accurate and sensitive (Hauser *et al.*, 1997), the need to measure mRNA abundance in plant tissues on the whole-genome scale has increased. This need lead to the development of methods such as differential display (Liang & Pardee, 1992; Welsh *et al.*, 1992) which is a low-stringency PCR-based method, and cDNA–amplified fragment length polymorphism (cDNA-AFLP; Bachem *et al.*, 1996). cDNA-AFLP offers the possibility to investigate entire transcriptomes of poorly characterized genomes in a high-throughput manner which has been widely used to identify differentially expressed genes involved in a variety of plant processes (Bachem *et al.*, 2001; Durrant *et al.*, 2000; Qin *et al.*, 2000). However, the sensitivity of cDNA-AFLP is limited by the ability of cDNA libraries to capture low-abundance transcripts. Serial analysis of gene expression (SAGE; Velculescu *et al.*, 1995) is another elegant technique that combines differential display and cDNA sequencing approaches, and it has the advantage of being quantitative. Unfortunately, SAGE is laborious, requires an extensive foundation of sequence information, and suffers from some of the same concerns regarding low-abundance transcripts as cDNA-AFLP. Other methods to study transcriptomes include cap analysis of gene expression (CAGE), massive parallel signature sequencing (MPSS) and suppression subtractive hybridization (SSH).

Within the last decade, the availability of large expressed sequence tag (EST) libraries and databases has enabled the development of genome-wide expression monitoring techniques such as cDNA and oligonucleotide microarrays. For both types, the principle is that DNA molecules or oligonucleotides corresponding to genes of which the expression is analyzed (the probes) are attached in an ordered fashion to a solid support such as a nylon membrane or a glass slide. Automation of microarray production with robotic spotters (cDNA microarrays) and in situ synthesis of oligonucleotides (oligonucleotide microarrays) enable the production of microarrays with up to a million oligos representing nearly all genes of the entire genome. In order to measure the relative abundance of the corresponding transcripts in a RNA preparation, the sample (either cDNA or RNA) is first labelled with a fluorescent

marker and then hybridized to the microarray. The intensity of the hybridization signal is a measure for the relative abundance of the corresponding mRNA in the sample. The transcriptome refers to the complete collection of expressed mRNAs. Thus, comparing the hybridization signals for different mRNA samples allows changes in mRNA levels to be determined under the specific conditions tested (for example infected vs. non-infected) for all the genes represented on the microarray.

cDNA microarrays, usually prepared directly from cDNA libraries, carry the advantage that they can be used in "two-color" co-hybridization experiments that allow for a direct comparison of transcript abundance in two mRNA samples simultaneously. On one hand, this strategy only allows for comparative expression profiling; on the other, it has been shown to reduce experimental variation that occurs between different microarrays (Aharoni & Vorst, 2002). Oligonucleotide microarrays are fabricated using photolithography, a microfluidic technology, which utilizes light to direct the synthesis of short oligonucleotides onto a matrix (Fodor *et al.*, 1991). This technology is more expensive due to the costs associated with oligonucleotide design and synthesis; on the other hand, it offers the possibility to produce extremely high-density microarrays with up 300,000 oligos/1.28 cm^2 (Lipshutz *et al.*, 1999). The probes on oligonucleotide microarrays are designed in order to represent unique gene sequences which reduces the occurrence of cross-hybridization between related gene sequences belonging to a certain gene family or with common functional domains.

Transcriptome analyses of host plants during their interactions with phytopathogenic bacteria has provided crucial insights into both plant defense responses and processes that result in disease. For example, in a study that used a variety of techniques to study the transcriptome (including SSH and cDNA microarrays), it has been shown that over-expression of the *R*-gene against *Pseudomonas syringae* pv. *tomato Pto* in tomato induces gene expression changes similar to those observed during immune responses in animals (Mysore *et al.*, 2003). To gain insights into the molecular basis of tomato resistance to *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) strains expressing the effector AvrRxv transcriptome analyses were performed. This resistance is governed by three, non-dominant resistance genes that participate with an additive effect in elicitation of the HR. The study revealed novel insights into signalling and cellular responses triggered in tomato plants by this avirulence factor (Bonshtien *et al.*, 2005). Other studies have obtained global transcript profiles of plants undergoing *R*-gene mediated responses toward identifying genes that might enhance resistance when expressed highly in genetically engineered or selectively bred plants (Verdier *et al.*, 2004). In crops such as rice, cassava, citrus or tomato, microarray analyses of

interactions with *Xanthomonas* spp. have revealed involvement of specific genes encoding cell-wall modifying proteins, protein kinases responsible for signalling pathways and genes triggered by pathogen-associated molecular patterns (PAMPs; Cernadas *et al.*, 2008; Gibly *et al.*, 2004; Li *et al.*, 2006b). For example, a number of genes were reported to be co-regulated during the interactions of rice with *X. oryzae* pv. *oryzae* (*Xoo*) and with *Magnaporthe grisea* (the fungal pathogen causing rice blast) indicating shared defence pathways (Li *et al.*, 2006b). In addition, a number of candidate genes involved in signal transduction co-locating with a major QTL for broad-spectrum *Xoo* resistance and a QTL for submergence tolerance on chromosome 5 have been identified in rice. This study indicated that submergence tolerance and broad-spectrum *Xoo* resistance share a common signalling system (Kottapalli *et al.*, 2007). In citrus, many genes associated with PAMP recognition were commonly modulated after *X. axonopodis* pv. *aurantifolii* and *X. axonopodis* pv. *citrii* infection (Cernadas *et al.*, 2008). Similar results were obtained in a study of interactions of *Arabidopsis* with *P. syringae*. Transcriptomes of different responses such as compatible interactions and incompatible responses (plants carrying either of two *R*-genes) and plants exhibiting non-host resistance to a strain that normally infects bean (incompatible interaction) were compared (Tao *et al.*, 2003). This study has revealed that overall strong similarity among the responses mediated by *R*-genes and in non-host resistance exist, despite large differences in efficiency and functionality of resistance mechanisms. The study also revealed that the differences among responses in incompatible and compatible interactions were largely quantitative.

1.5.4 Marker-assisted selection for disease resistance

Ideally, the gained knowledge from QTL studies and transcriptome analyses can be used to develop markers for marker assisted selection (MAS). MAS is defined as the process whereby a marker (morphological, biochemical or based on a DNA polymorphism) is used for selection of genetic determinants of traits of interest such as yield, disease resistance, abiotic stress tolerance or quality. Due to genetic linkage, QTL mapping and the identification of candidate genes for disease resistance can be used to develop DNA markers that can detect the presence of allelic variation in genes underlying the desired trait. When allelic variation relies on allelic variation of, for example, single nucleotide polymorphism (SNP) markers within identified candidate genes, it is then referred to as a functional marker (Andersen & Lübberstedt, 2003). Functional markers are considered to be more efficient because they cannot uncouple from the desired trait. By using DNA markers and functional markers in plant breeding, MAS can substantially enhance efficiency and precision of the breeding process. On one hand, MAS may be carried out for transferring target gene(s) from one

genetic background to another using tightly linked markers (foreground selection). On the other hand, MAS is also carried out to quickly recover recurrent parent genome in backcross breeding using a large number of markers with whole-genome coverage called background selection (reviewed in Collard & Mackill, 2008). Depending on the trait, screening for DNA markers is not only simpler and less time consuming than phenotypic selection, it is also possible to select at the seedling stage for the desired trait that may not be expressed until later stages. In general, MAS is more integrated in line breeding schemes of crops such as wheat and rice and in some cases are even used to substitute conventional phenotypic selection (Huang *et al.*, 1997; Liu & Anderson, 2003). However, it has been assumed that once a gene or QTL that contributes to the desired trait such as disease resistance is identified and a molecular marker is developed, the marker can be directly used for MAS. This trend has changed widely and QTL and functional marker validation in different genetic backgrounds and especially in important breeding material has shown to be required in many cases (Langridge *et al.*, 2001). The steps involved are (1) QTL and functional marker confirmation: reproducing the accuracy of results from a primary QTL mapping or functional genomics study, (2) QTL and functional marker validation: verification that a QTL/gene is effective in different genetic backgrounds and different mapping populations and (3) marker validation: testing the level of polymorphism of most tightly-linked markers in vicinity of the target locus and also testing the reliability of markers to predict the desired phenotype (Collard & Mackill, 2008).

1.6 Recent advances in the characterization of bacterial wilt resistance in Italian ryegrass

Previous efforts to investigate qualitative and quantitative inheritance of *Xtg* resistance have included the development of a high density genetic linkage map for Italian ryegrass (*L. multiflorum*) from a pseudo-testcross family and QTL analyses (Studer *et al.*, 2006). The linkage map consists of seven linkage groups and 367 AFLP and 51 SSR markers. QTL analyses demonstrated that bacterial wilt resistance was controlled by one major QTL on linkage group (LG) 4 explaining between 43 and 84% of the total phenotypic variance (Studer *et al.*, 2006). Phenotypic traits that result in the identification of one single QTL explaining such a high percentage of the total observed phenotypic variance have often been shown to be controlled by one or only a few major *R*-genes (Mutlu *et al.*, 2005; Mutlu *et al.*, 2006; Verdier *et al.*, 2004; Yang & Francis, 2005). These results led to the conclusion that *Xtg* resistance could be controlled qualititatively by major *R*-genes. Therefore, in order to find further

indications for race-specificity, screening for genetic diversity of different *Xtg* isolates and assessment of virulence of these isolates was performed. 29 different *Xtg* isolates were characterized in detail using 16S rDNA sequencing and AFLP analysis (Kölliker *et al.*, 2006). Interestingly, AFLP analyses revealed a high level of genetic similarity of all isolates identified as *Xtg* isolates, despite some moderate differences in virulence. These results indicated that *Xtg* isolates with specific virulence and/or avirulence may not have evolved yet. This could be explained by the fact that in out-breeding species such as ryegrasses, where cultivars are population based, avirulent strains against specific cultivars are rarely observed (Kimbeng, 1999). In contrast, in the case of the causal agent of crown rust, *Puccinia coronata* f. sp. *lolii*, some major genes or quantitative trait loci (QTL) contributing considerably to resistance against this disease have been identified (Dumsday *et al.*, 2003; Thorogood *et al.*, 2001) and evidence for the existence of races has been demonstrated (Aldaoud *et al.*, 2004). Therefore, a better understanding of the interaction between different *Xtg* isolates and well-characterized *L. multiflorum* genotypes is necessary.

Nevertheless, for defining the roles of individual genes in *Xtg* resistance, classical quantitative genetics has not provided efficient tools. Therefore, transcriptome analyses of a resistant *L. multiflorum* genotype inoculated with *Xtg* using cDNA-amplified fragment length polymorphism (AFLP) was performed (Rechsteiner *et al.*, 2006). This study identified a number of differentially expressed transcript derived fragments (TDF) at different time points after inoculation. TDF sequences of particular interest with annotations associated to defense responses were the ethylene-responsive binding protein (ERBP) transcription factors, a transforming growth factor (TGF-β) receptor-interacting protein and a terpene synthase. However, the number of identified TDFs was limited and sequencing of the TDFs revealed only short reads. This limited the usefulness of the results for further investigations based on candidate genes of the genetic control. In addition, the cDNA-AFLP analysis could not take into account the high genetic diversity of *L. multiflorum* genotypes present in populations and cultivars due to the out-breeding reproduction system of the species. Therefore, a more comprehensive transcriptome analysis is needed to gain a more detailed insight into genes and pathways involved in defense responses against *Xtg*.

1.7 Objectives

Although conventional breeding has led to cultivars of *L. multiflorum* with considerable levels of *Xanthomonas* resistance, it is difficult to obtain further breeding progress solely by means of recurrent phenotypic selection because susceptible individuals still occur in advanced breeding germplasm. In order to complement the current breeding schemes with MAS, molecular markers closely linked to resistance genes and QTL are needed. However, the development of MAS tools presumes a detailed knowledge on the number of resistance genes (*R*-genes) and inheritance mode (qualitative or quantitative) of these *R*-genes and QTL. Despite previous results that have implied qualitative *Xtg* resistance in *L. multiflorum*, the exact mechanism of resistance has not completely been clarified. Therefore, the first aim was to elucidate the existence of qualitative resistance in the *L. multiflorum-Xtg* interaction. In addition, genetics and classical breeding has not allowed for a determination of gene functions and pathways involved in resistance mechanisms of resistant individuals. Transcriptome analyses using microarrays are a promising tool to elucidate host defense responses and identify genes linked to disease resistance. By means of such transcriptome analyses and functional genomics studies, functional markers could be identified which may be used for MAS. Therefore, the second aim was to identify genes and pathways involved in *Xtg* resistance. On the other hand, focusing more on the pathogen instead of the host, another promising option for resistance mechanism identification is understanding in more detail bacterial multiplication inside its host. Functional characterization of the genes encoding virulence factors of the pathogen inside the host plants would potentially allow targeted *R*-gene identification. Therefore, the third aim was to identify genes encoding virulence factors in *Xtg*. The general aim of this thesis was to characterize in more detail the *L. multiflorum–Xtg* interaction in order to identify candidate genes for the development of molecular markers that are applicable to Italian ryegrass breeding. The more specific objectives to reach this overall goal were:

- to elucidate the existence of race-specific resistance of selected *L. multiflorum* genotypes to different bacterial isolates
- to study molecular marker-trait associations in a broad range of different *L. multiflorum* genotypes in order to facilitate MAS in Italian ryegrass breeding.
- to identify candidate genes for bacterial wilt resistance by means of transcriptome analyses with a resistant *L. multiflorum* genotype
- to compare the transcriptomes of a resistant and a susceptible *L. multiflorum* genotype
- to identify virulence factors and genes responsible for pathogenicity in *Xtg*.

2 Phenotypic and molecular genetic characterization indicate no major race-specific interactions between *Xanthomonas translucens* pv. *graminis* and *Lolium multiflorum*

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2.1 Abstract

Bacterial wilt of forage grasses, caused by the pathogen *Xanthomonas translucens* pv. *graminis* (*Xtg*), is a major disease of forage grasses such as Italian ryegrass (*Lolium multiflorum* Lam.). The plant genotype–bacterial isolate interaction was analyzed to elucidate the existence of race-specific responses and to assist the identification of plant disease resistance genes. In a greenhouse experiment, 62 selected plant genotypes were artificially inoculated with six different bacterial isolates. Significant differences in resistance were observed among *L. multiflorum* genotypes ($P<0.001$) and in virulence among *Xtg* isolates ($P<0.001$) using the area under the disease progress curve (AUDPC). No significant genotype–isolate interaction ($P>0.05$) could be observed using linear regression modelling. However, additive main effects and multiplicative interaction effects (AMMI) analysis revealed five genotypes, which did not cluster close to the origin of the biplot, indicating specific interactions between these genotypes and some bacterial isolates. Simple sequence repeat (SSR) markers were used to identify marker-resistance associations using the same plant genotypes and bacterial isolates. The SSR marker NFA027 located on linkage group (LG) 5 was significantly associated with bacterial wilt resistance across all six bacterial isolates and explained up to 37.4% of the total variance of AUDPC values. Neither the inoculation experiment nor the SSR analyses revealed major host genotype–pathogen isolate interactions, thus suggesting that *Xtg* resistance, so far observed, is effective across a broad range of different bacterial isolates and plant genotypes.

2.2 Introduction

Bacterial wilt of forage grasses is caused by the pathogen *Xanthomonas translucens* pv. *graminis* (Egli *et al.* 1975) Vauterin *et. al.* 1995. It is one of the most important diseases of forage grasses in temperate regions, and so far four different pathovars of *X. translucens* have been described to infect forage grasses. *X. translucens* pv. *graminis* (*Xtg*) infects a broad range of forage grasses including ryegrasses, while the other pathovars are mostly restricted to one genus (Egli & Schmidt, 1982). Bacterial wilt is estimated to account for annual forage yield losses of 10–15% in cultivated grassland; however, depending on cultivar susceptibility, *Xtg* may also lead to complete yield losses in pure and mixed stands (Suter *et al.*, 2005).

Xtg enters grass leaves and roots through wounded tissue, multiplies in the xylem and causes symptoms including wilting of leaves, yellow stripes along the vascular tissue and withering of entire tillers. In severe cases, the plants die within a few days after inoculation. Since treatments with antibiotics are undesired in forage production, and both the disinfection

of mowing equipment and induced systemic resistance with epiphytic bacteria have not yielded satisfactory results (Schmidt, 1988c), resistance breeding is currently the only applicable and accepted measure to prevent substantial yield losses. So far, resistance breeding programs have been based on artificial seedling inoculation in the greenhouse and targeted recurrent phenotypic selection. Although considerable progress has been achieved, further advances in breeding programs have often stagnated after several cycles of recurrent selection, and susceptible individuals have still been observed in advanced populations (Michel, 2001). Italian ryegrass (*Lolium multiflorum* Lam.) is a major forage crop for hay and silage production, and therefore particularly prone to infection through contaminated mowing equipment. Understanding the genetic basis of *Xtg* resistance is fundamental for the further improvement of disease resistance and for the development of molecular tools, which may help to enhance phenotypic selection through marker assisted selection (MAS).

Xanthomonas resistance has been extensively studied in rice where *X. oryzae* pv. *oryzae* (*Xoo*) causes bacterial blight. At least 33 race-specific genes conferring resistance to *Xoo* have been identified and seven have been cloned (reviewed in Niño-Liu *et al.*, 2006; White & Yang, 2009). Knowledge on this interaction may provide a suitable model for *Xanthomonas* resistance in *L. multiflorum* due to the syntenic relationships between species among the members of the grass family (Devos, 2005). *Xanthomonas* resistance genes in rice are mostly based on race-specific recognition of effector proteins secreted by pathogens. Some of these race-specific resistance genes are only effective against one or a few *Xoo* (e.g. *Xa1*), whereas others condition resistance to a wide spectrum of different isolates (e.g. *Xa21* and *Xa23*). Race-specific (or qualitative) resistance is usually conferred by a single resistance gene, and therefore may exert high selection pressure on pathogen populations, which eventually evolve to overcome resistance. In contrast to *Xoo* resistance, no race-specific resistance genes have been found that are effective against *X. o.* pv. *oryzicola* (*Xoc*) isolates. *Xoc* resistance has been shown to be inherited quantitatively by eleven quantitative trait loci (QTL) that together explained 84.6% of the total phenotypic variance (Tang *et al.*, 2000). Quantitative resistance controlled by various genes, is considered to be more durable when compared to race-specific, qualitative resistance; however it carries the disadvantage of providing only partial resistance and being less effective when conditions are particularly favourable for the pathogen (Geiger & Heun, 1989).

In *L. multiflorum*, QTL analysis of a pseudo-testcross family has revealed that bacterial wilt resistance was controlled by one major QTL on linkage group (LG) 4 explaining up to 84% of the total phenotypic variance (Studer et al. 2006). In addition to this major QTL,

three minor QTL on LG 1, 5 and 6 were identified, explaining between 2.9 and 7.4% of the total phenotypic variance. In general, the existence of major QTL is often associated with the presence of one or only a few major resistance genes (Mutlu *et al.*, 2005), and race-specificity is likely to develop for such genes. On the other hand, cultivar–isolate interactions of five commercially available *L. multiflorum* cultivars inoculated with four different *Xtg* isolates have revealed that the cultivar–isolate interaction was not significant (Michel, 2001). However, ryegrass cultivars are multi-genotype populations, and therefore these results have not allowed for a conclusion concerning race-specificity at the genotype level. Thus, the genetic control of *Xtg* resistance and the presence of race-specificity in the *L. multiflorum–Xtg* interaction remain unclear.

Recently, additive main effects and multiplicative interaction (AMMI) analyses and the resulting biplot have been shown to be effective tools to understand and describe complex host–pathogen interactions even if large variability within host resistance or pathogen virulence is expected (Yan & Falk, 2002). AMMI is a hybrid analysis that combines additive and multiplicative components of a two-way data structure. The biplot that results from the calculation of principal component analysis (PCA) scores displays the host-by-pathogen interaction term and facilitates the interpretation of race-specific resistances.

The association of molecular markers and phenotypic traits such as disease resistance may be investigated in genetically diverse plant populations to allow for the development of diagnostic markers. In this approach, molecular marker alleles with frequencies correlating with the attributes of the phenotype are identified. For the selection of alleles to be included in the linear regression model, least absolute shrinkage and selection operator (LASSO) subset selection is an efficient dimension-reduction technique, which produces a stable and interpretable model (Tibshirani, 1996). In addition to the identification of alleles associated with bacterial wilt resistance, significant associations would also reveal the existence of race-specific interactions.

The aims of the present study were to elucidate the existence of race-specificity in different *L. multiflorum* genotype–*Xtg* isolate combinations and to perform a marker-trait association analysis. A detailed investigation of the genotype–isolate interaction and the identification of molecular markers correlated with *Xtg* resistance may provide a starting point for the development of markers for marker-assisted breeding tools.

2.3 Materials and Methods

2.3.1 Bacterial isolates and plant material

Six bacterial isolates collected in Switzerland and previously characterized for genetic diversity and virulence (*Xtg*3, *Xtg*5, *Xtg*8, *Xtg*9, *Xtg*19 and *Xtg*29; Kölliker *et al.*, 2006) were selected in order to represent a broad genetic diversity and geographical distribution of original sampling sites. Bacteria were stored at -80ºC in GYC (glucose 20 g/L, yeast extract 10 g/L, $CaCO_3$ 20 g/L) broth containing 150 ml/L glycerol. Sixty-two plant genotypes selected from 12 cultivars, ecotypes or breeding populations were used for this study (populations *Lm*A–*Lm*L). The genotypes of population *Lm*A are F_2 progeny of a mapping population segregating for bacterial wilt resistance (Studer *et al.*, 2006), genotypes of population *Lm*B are Syn1 progeny of a polycross with nine elite genotypes, genotypes of the population *Lm*C are from a cultivar candidate from Agroscope Reckenholz-Tänikon, *Lm*J, *Lm*K and *Lm*L are individuals from different commercially available cultivars (Turilo, Adret and Axis, respectively), and genotypes of the populations *Lm*D, *Lm*E, *Lm*F, *Lm*G, *Lm*H, *Lm*I are F_2 progeny of ecotypes collected in different parts of Switzerland. Population *Lm*A consisted of 12 genotypes, populations *Lm*B, *Lm*C, *Lm*J and *Lm*L consisted of 5 genotypes, populations *Lm*D, *Lm*E, *Lm*F, *Lm*G, *Lm*H and *Lm*I consisted of 4 genotypes, and population *Lm*K consisted of 6 genotypes. The genotypes were selected based on a pre-screening in order to represent different levels of resistance and susceptibility to *Xtg* by using a mixture of bacterial isolates (data not shown).

2.3.2 Experimental design and disease scoring

All *L. multiflorum* genotypes were clonally propagated by separating single tillers, which were transferred into soil-filled pots and grown in a greenhouse. Assessment of bacterial wilt symptoms was performed using four replications per genotype–isolate combination in a completely randomized block design. Replications were assigned to four sequentially grown blocks consisting of 372 plants each resulting from all possible combinations of 62 *L. multiflorum* genotypes with six *Xtg* isolates. After eight weeks, the plants were inoculated with the six different *Xtg* isolates using the leaf clipping technique described by Kölliker *et al.* (2006). The inoculated plants were allowed to regrow in a greenhouse at average temperatures of 20ºC/18ºC (day/night), under long day conditions (16 h light, [>100µE/m2s1]) at about 70% relative humidity. Scoring of bacterial wilt symptoms was performed on whole plants 15, 21 and 28 days after inoculation according to a scale ranging from 1 to 9. Disease scores were 1 = no symptoms, 2 = wilting or withering next to the cutting area, 3 = one entire leaf is

wilting or is completely withered, 4 = half of the leaves and tillers are wilting, 5 = all leaves and tillers are affected, 6 = intermediate score, 7 = all leaves and tillers are affected and half of them are withering, 8 = plant is dried, base of tillers is still green, 9 = plant is dead, no green parts. Twenty-eight days after inoculation, the plants were cut with sterilized scissors and disease symptoms were again scored 21 days later (49 days after inoculation).

In addition, a control-experiment was performed to test whether plants showing weak disease symptoms could be distinguished from control-treated plants (inoculation with NaCl solution). Therefore, the three isolates *Xtg*3, *Xtg*9 and *Xtg*19 were used to inoculate a selection of 13 different *L. multiflorum* genotypes in four replications grown simultaneously. None of the control-treated plants showed disease symptoms (score =1), while disease scores of *Xtg* inoculated plants ranged from 2 to 9 and revealed significant differences among *L. multiflorum* genotypes in a Wilcoxon Sum rank test (P<0.001).

2.3.3 Data analyses and statistics

Area under the disease progress curve (AUDPC) values were calculated for each inoculated plant from the scores obtained at 15, 21 and 28 days after inoculation following the formula ($AUDPC = \sum_i [(Y_i + Y_{i-1}) \times (t_i - t_{i-1})] / 2$) where Y_i is the disease score at time point i and t_i is the number of days after inoculation. Plants were assigned to three categories according to their AUDPC values: Resistant (AUDPC values ≤39; disease symptoms were never described with a score higher than 3), moderately susceptible (AUDPC values between 39 and 60; disease symptoms were never described with a score higher than 6), and susceptible (AUDPC values>60). AUDPC values were used for all ANOVA and AMMI analyses.

The scores obtained at 49 days after inoculation (49d scores) showed an asymmetric distribution with many plants being either dead (score = 9) or healthy (scores = 2 or 3). The normal-score-transformation suggested by Tilquin (2003) was used to transform scores towards a normal distribution for subsequent ANOVA. AUDPC values were fitted to a linear regression model including the factors replication, *L. multiflorum* genotype, *Xtg* isolate, and the interaction *L. multiflorum* genotype and *Xtg* isolate. The factor population was not included in the model, because *L. multiflorum* genotypes were not chosen at random and therefore do not represent the entire group.

Interactions between *L. multiflorum* genotypes and pathogen isolates were studied by applying an AMMI model with additive effects for plant genotypes (G) and isolates (I) and a multiplicative term for G–I interaction (De Mendiburu, 2009). The AMMI analysis combines the ANOVA and the singular value decomposition (SVD) and has been explained in detail by

Gollob (1968). In this analysis, multiplicative effects for G–I are fitted by principal component analysis (PCA). The additive interaction in the ANOVA model is obtained by multiplication of genotype PC scores by isolate PC scores. A reduction of PCs to one or two axes (PC1 and PC2) is used to plot the interaction effect via the PC scores for genotypes and isolates. The AMMI model is described as:

$$Y_{ij} = \mu + g_i + e_j + \sum_{k=1}^{n} \lambda_k £_{ik} Y_{jk} + R_{ij}$$

where Y_{ij} is the AUDPC value of the i^{th} genotype inoculated with the j^{th} isolate, g_i is the mean AUDPC value of the i^{th} genotype minus the grand mean, e_j is the effect of the j^{th} isolate, λ_k is the square root of the eigenvalue of the PCA axis k, $£_{ik}$ and Y_{jk} are the principal component scores for the PCA axis k of the i^{th} genotype and the j^{th} isolate and R_{ij} is the residual. In order to facilitate visualization of the relationships among isolates and genotypes, a polygon was drawn on the genotypes farthest from the biplot origin such that all other genotypes were contained within the polygon.

2.3.4 Molecular marker and association analysis

Genomic DNA was extracted from lyophilized plant leaves using the Corbett x-tractor Gene roboter (Qiagen) and the Nucleospin 96 plant kit (Macherey-Nagel) following the manufacturers recommendations. DNA concentrations and purities were assessed with a Nanodrop spectrophotometer (Thermo Fisher Scientific) and the ND-1000 software. The simple sequence repeat (SSR) primers used for PCR amplification were selected to be localized on the same LGs where QTL for bacterial wilt resistance were identified previously in glasshouse and field experiments (Studer *et al.*, 2006; Table 2.1).

Table 2.1 Names, sequences and references of simple sequence repeat (SSR) markers used to genotype 62 *Lolium multiflorum* plants differing in resistance to bacterial wilt. Linkage groups and positions were determined in the *L. multiflorum* mapping population as described by Studer et al. (2006).

Locus	Linkage group	Position (cM)	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Reference
G01_079	1	14.9	GTCACTCCCATTCCCTACGA	GATAGCTGATAGCACCGAACG	Studer *et al*., (2008)
LMgSSR04-09	1	32.7	ATCGGACACTGGTTCCGCAT	TTGTTGTTGCCGGCTTCGTA	Hirata *et al.,* (2000)
G02_037	1	49.8	AGGCGTCACAGTTGGAAGAG	TCCTTTTATCGCATTCACGA	Studer *et al*., (2008)
G01_031	1	50.7	ATGAACACCCAGGATTGGAA	TGTATGCAGCTCAGGGTTTG	Studer *et al*., (2008)
G06_043	4	0.0	CTGGCTTTCCTCTCCCTTTC	GAAGAGGGTGGAGACGATGA	Studer *et al*., (2008)
G04_034	4	34.4	TGACCTCAGCTACGACGACA	GCCTCTCTCCCGTTTCCTAT	Studer *et al*. (2008)
G05_122	4	56.8	AGCACAAAGAAGCTCCCAAA	CGACCATGCTGGTGATGTAG	Studer *et al*., (2008)
G04_085	4	58.2	AGCACAAAGAAGCTCCCAAA	CGACCATGCTGGTGATGTAG	Studer *et al*., (2008)
G04_045	4	74.6	ACCCTACCCTCCTCCTTCCT	GTCTTGACGTCCCAGAGCTT	Studer *et al*., (2008)
G06_089	4	79.9	AGATGGGAGGTGATCAGGTG	GAATCTTGGCAGAAGCCCTA	Studer *et al*., (2008)
G05_121	4	83.4	CGTCTTCACCAAGATCGACA	TTGCGATCCATGCACTATACA	Studer *et al*., (2008)
G03_013	4	89.5	CAGCTGTCCTCTGCTCACAA	GCAGGTGATACATCGCACAT	Studer *et al*., (2008)
G05_139	4	103.7	GGTACGGACTCTCCCTCTCC	AGCTTGGCTATGTTCGCATT	Studer *et al*., (2008)
NFA027	5	6.5	CGAGGTCTCAATCCTCCATT	GTTTCTTGACAGAGACGACGACGACAT	Saha *et al.,* (2004)
NFA059	5	9.9	GTCGCCGGAGAAGAGAAGAG	GTTTCTTAACGCTAGCCGTGATGACTT	Saha *et al.,* (2004)
G05_044	5	16.5	GACCGATTGGAACCAACAAC	CGATGCTTTCAGCGGTTAAT	Studer *et al*., (2008)
G07_056	6	67.8	CAAAGAAGTCACGCACCAAA	GCTGGTGTAGCAGATGAGCA	Studer *et al*., (2008)
LMgSSR03-04	6	80.9	CAGATGGGCAGTTGCCACTG	GTATTGTACACACAAGCATATTGGCG	Hirata *et al.,* (2000)
B1A8	6	83.5	GACTTTCAGGCATCGGTCAT	CCCAGCTCCATTCTTAATGC	Lauvergeat *et al*., (2005)
G04_056	6	91.1	CAAGGGTGTGGCGATTAACT	ATCGGCATCATCATCAGACA	Studer *et al*., (2008)

PCR reactions were conducted in a total volume of 20 µl containing 10 ng of genomic DNA, 4 µl of 5x PCR buffer, 0.4 µM labelled (FAM, HEX or NED) forward and unlabelled reverse primer, 2 mM $MgCl_2$, 0.2 mM of each dNTP and 0.75 U GoTaq® Flexi DNA Polymerase (Promega). PCR conditions were as follows: initial denaturation for 5 min at 94ºC, followed by 12 cycles of touchdown PCR consisting of 30 s at 94ºC, 1 min at 72ºC (decreasing by 1ºC per touchdown cycle) and 1 min at 72ºC. After 30 cycles of 30 s at 95ºC, 1 min at 60ºC, 1 min at 72ºC and a final extension of 5 min at 72ºC. PCR fragments were separated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) and analyzed using the ROX HD400 standard (Applied Biosystems) and the GeneMarker® software version 1.51 (SoftGenetics). SSR alleles were scored for presence (1) or absence (0) and entered into a binary matrix for each genotype. In order to assess genetic variation within and between populations, an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was performed with the software Arlequin (Schneider *et al.*, 2000).

Based on this matrix and the AUDPC values assessed in the greenhouse, all alleles were submitted to a least absolute shrinkage and selection operator (LASSO) analysis (Tibshirani, 1996). This shrinkage method represents an efficient tool for subset selection of parameters to be included in the regression model. LASSO subset selection was carried out for each bacterial isolate separately. The alleles selected by LASSO were included in a linear regression model with the log-transformed AUDPC values for each plant genotype. Alleles with a significant effect on bacterial wilt resistance were identified using ANOVA.

All statistical analyses were performed with the R statistical software (The R Development Core Team, 2008) using the packages: stats, graphics, coin (Hothorn *et al.*, 2006), agricolae (De Mendiburu, 2009) and lars (Hastie & Efron, 2007).

2.4 Results

2.4.1 Data structure and distribution

No complete resistance was observed on any plant genotype and all scores between 2 and 9 were used to describe bacterial wilt symptoms. Average disease scores ranged from 2.67±0.97 (15 days after inoculation) to 3.39±1.16 (21 days after inoculation) and reached a maximum value of 3.66±1.37 (28 days after inoculation). At 28 days after inoculation, the plants were cut using sterile scissors and allowed to regrow for 21 days. The resulting average 49d-scores were at 2.86±1.78. The AUDPC values varied greatly among some *L. multiflorum* genotypes (Fig. 2.1). Comparison of the mean AUDPC values across all *Xtg* isolates revealed that genotype *Lm*K-01 was the most susceptible genotype with a mean AUDPC value of 83.72,

whereas genotype *Lm*G-04 was the most resistant genotype with a mean AUDPC value of 30.06 (Fig. 2.1). The bacterial isolates also varied significantly in their virulence. *Xtg*9 and *Xtg*8 were the most virulent bacterial isolates, whereas *Xtg*19 and *Xtg*3 were the least virulent *Xtg* isolates. *Xtg*5 and *Xtg*29 were intermediately virulent.

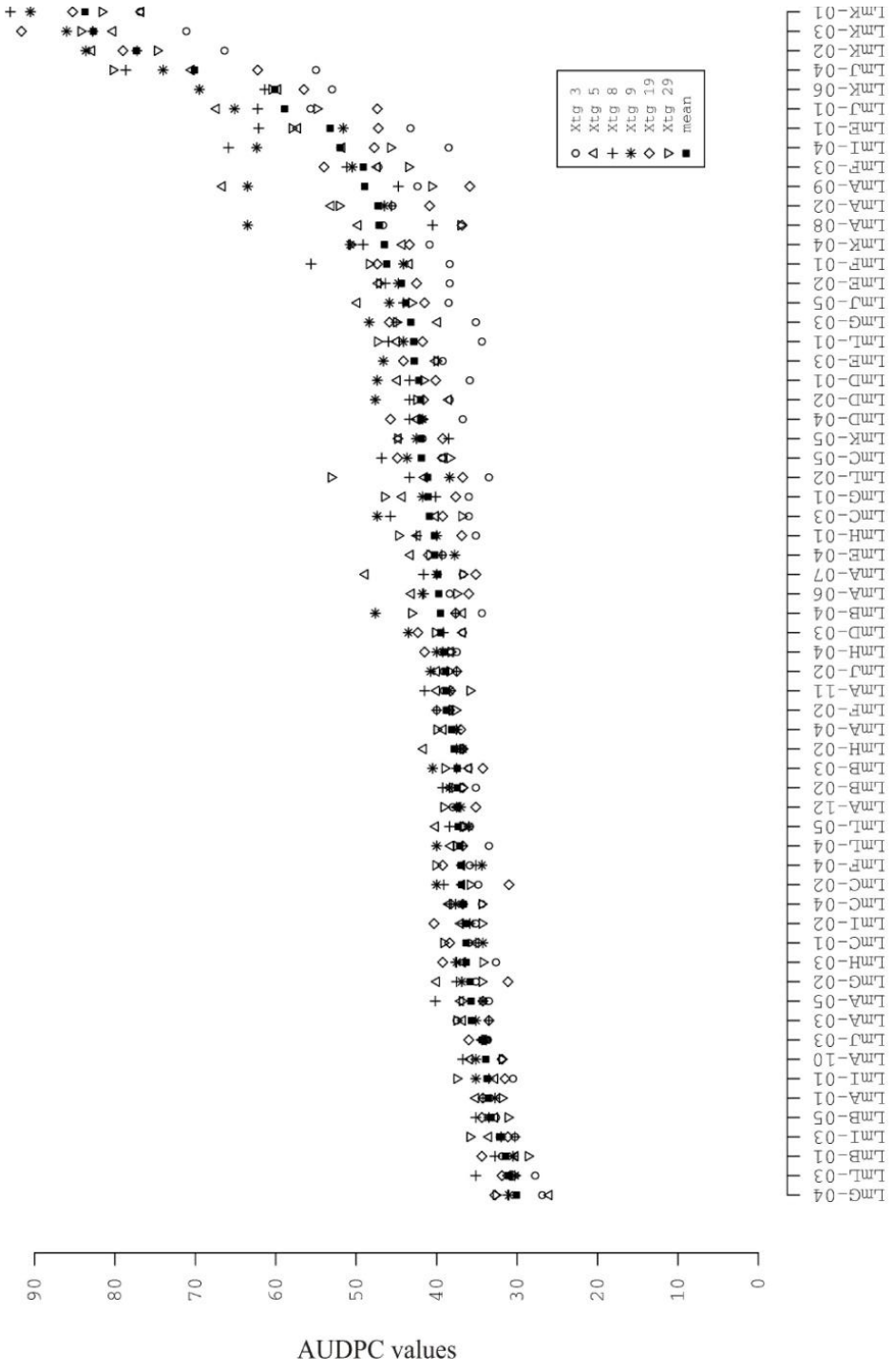

Figure 2.1 Mean area under the disease progress curve (AUDPC) values from four replications for each of 62 *Lolium multiflorum* genotypes inoculated with six different *Xanthomonas translucens* pv. *graminis* isolates (standard error = 0.37). The AUDPC values are ranked according to mean AUDPC values calculated across all *Xtg* isolates. left: most resistant genotype, right: most susceptible genotype.

2.4.2 Regression modelling and race-specificity

Significant differences of the AUDPC values among *L. multiflorum* genotypes ($P<0.001$) were observed using ANOVA for the days 15, 21 and 28 days after inoculation (Table 2.2) and the normal-transformed 49d-scores (Table 2.3). In addition, the factors replication and *Xtg* isolate ($P<0.001$) had considerable influence on the observed variance of AUDPC values. The factors included in the ANOVA explained 73% of the total variance of AUDPC values. *F*-values of replication and *L. multiflorum* genotype were high (62.94 and 39.18), underlining their strong contribution to variance effects (Table 2.2). The interaction of bacterial isolate and plant genotype was not significant in the linear regression analysis of AUDPC values, and the *F*-value was very low ($P>0.05$). Furthermore, no significant influence of the *Xtg–L. multiflorum* interaction was observed with ANOVA of normal-transformed 49d-scores ($P>0.05$; Table 2.3).

Table 2.2 Analysis of variance for area under disease progress curve (AUDPC) values of 62 *Lolium multiflorum* genotypes inoculated with six *Xanthomonas translucens* pv. *graminis* (*Xtg*) isolates.

Source of variation	DF	Mean squares	*F*-statistics
Replication	3	4725	62.94 ($P<0.001$)
L. multiflorum genotype	61	2941	39.18 ($P<0.001$)
Xtg isolate	5	999	13.31 ($P<0.001$)
L. multiflorum genotype–*Xtg* isolate	305	63	0.95 ($P=0.97$)
Residuals	1113	75	

Table 2.3 Analysis of variance of normal-transformed disease scores obtained at 49 days after inoculation of 62 *Lolium multiflorum* genotypes inoculated with six *Xanthomonas translucens* pv. *graminis* (*Xtg*) isolates.

Source of variation	DF	Mean squares	*F*-statistics
Replication	3	2.43	7.53 (P<0.001)
L. multiflorum genotype	61	6.75	20.87 (P<0.001)
Xtg isolate	5	3.05	9.43 (P<0.001)
L. multiflorum genotype–*Xtg* isolate	305	0.33	1.03 (P=0.36)
Residuals	1113	0.32	

Using the AUDPC value based classification into resistant (R), moderately susceptible (MS) and susceptible (S) genotypes, eleven (17.7%) *L. multiflorum* genotypes were resistant (R) across all bacterial isolates, four (6.4%) were moderately susceptible (MS) across all bacterial isolates, and three (4.8%) were susceptible (S) across all bacterial isolates (Table 4). The other 44 (71.1%) *L. multiflorum* genotypes showed variable resistance to one or more bacterial isolates, but there were only two genotypes (3.2%) that were classified as resistant (R) to one or more bacterial isolates and at the same time were classified as susceptible (S) to one or more other bacterial isolates. Further, there were three genotypes, that showed significantly different AUDPC values across different isolates according to a pairwise t-test (P<0.05). This analysis implies that some genotype–isolate specific interactions could exist. Therefore, the interaction between pathogen isolates and plant genotypes was evaluated by AMMI and visualized using a biplot (Fig. 2.2).

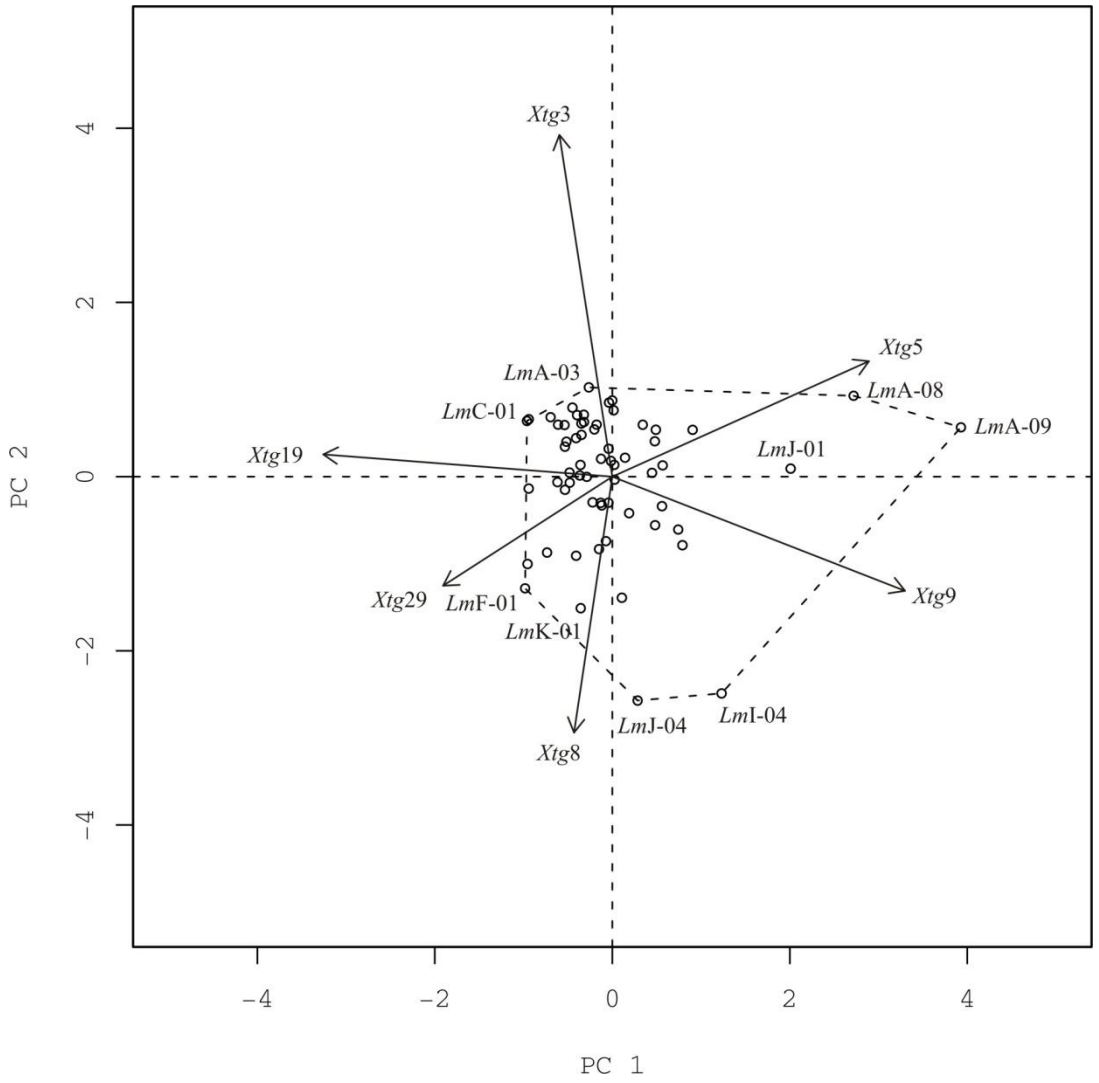

Figure 2.2 Biplot of the first two interaction principal component (PC) axes scores derived from additive main effects and multiplicative interaction effects (AMMI) analysis of area under the disease progress curve (AUDPC) values of 62 *Lolium multiflorum* genotypes (*Lm*) inoculated with six *Xanthomonas translucens* pv. *graminis* isolates. The dashed line connects the *L. multiflorum* genotypes located on the vertices of the polygon. *L. multiflorum* genotypes not clustering close to the origin (PC1 and PC2 scores: -1.5< or >1.5) are indicated with names. Arrows indicate direction and distance from the origin of PC scores of the *Xtg* isolates.

The first 2 principal component axes of the biplot accounted for 35.7% (PC1) and 26.2% (PC2) of the total variation of the genotype–isolate interaction. In this biplot, genotypes *Lm*A-08, *Lm*A-09, *Lm*I-04, *Lm*J-04, *Lm*F-01, *Lm*C-01 and *Lm*A-03, were located on the vertices of the polygon, indicating differential response to some specific isolates. On the other hand, genotypes *Lm*A-03, *Lm*F-01 and *Lm*C-01 were located very close to the origin of the biplot indicating no differential response. Therefore, only five out of 62 genotypes (*Lm*J-04, *Lm*I-04, *Lm*A-09, *Lm*A-08 and *Lm*J-01) that did not cluster close to the origin (PC1

and PC2: -1.5< or >1.5) were considered to show potential race-specific resistance. For example, genotypes *Lm*A-09 and *Lm*A-08 were especially susceptible to the isolates *Xtg*5 and *Xtg*9, but only moderately susceptible to the other isolates, while genotype *Lm*I-04 was particularly susceptible to isolates *Xtg*8 and *Xtg*9 but also only moderately susceptible to the other isolates (Table 2.4).

Table 2.4 Classification of 62 *Lolium multiflorum* genotypes according to their resistance to six bacterial *Xanthomonas translucens* pv. *graminis (Xtg*) isolates. Genotype–isolate combinations with different letters showed significantly different area under disease progress curve (AUDPC) values according to a pairwise t-test (P<0.05) determined on four replications per genotype–isolate combination. *P*-value adjustment was performed according to Holm (1979).

Plant genotypes	Bacterial isolates					
	*Xtg*3	*Xtg*5	*Xtg*8	*Xtg*9	*Xtg*19	*Xtg*29
*Lm*A-01	R	R	R	R	R	R
*Lm*A-02	MS	MS	MS	MS	MS	MS
*Lm*A-03	R	R	R	R	R	R
*Lm*A-04	R	MS	R	R	R	MS
*Lm*A-05	R	R	MS	R	R	R
*Lm*A-06	R	MS	MS	MS	R	R
*Lm*A-07	R [a]	MS [b]	MS [ab]	MS [ab]	R [a]	R [ab]
*Lm*A-08	MS	MS	MS	S	R	R
*Lm*A-09	MS	S	MS	S	R	MS
*Lm*A-10	R	R	R	R	R	R
*Lm*A-11	MS	MS	MS	R	R	R
*Lm*A-12	R	R	R	R	R	MS
*Lm*B-01	R	R	R	R	R	R
*Lm*B-02	R	R	MS	R	R	R
*Lm*B-03	R	R	R	MS	R	R
*Lm*B-04	R	R	MS	R	R	MS
*Lm*B-05	R	R	R	R	R	R
*Lm*C-01	R	R	R	R	R	MS
*Lm*C-02	R	R	MS	MS	R	R
*Lm*C-03	R [a]	MS [ab]	MS [b]	MS [b]	MS [ab]	R [a]
*Lm*C-04	R	R	R	R	R	R
*Lm*C-05	MS	MS	MS	MS	MS	R
*Lm*D-01	R	MS	MS	MS	MS	MS
*Lm*D-02	R	R	MS	MS	MS	MS
*Lm*D-03	R	R	MS	MS	MS	MS
*Lm*D-04	R	MS	MS	MS	MS	MS
*Lm*E-01	MS	MS	S	MS	MS	MS
*Lm*E-02	R	MS	MS	MS	MS	MS
*Lm*E-03	MS	MS	MS	MS	MS	MS
*Lm*E-04	MS	MS	MS	R	MS	MS
*Lm*F-01	R	MS	MS	MS	MS	MS
*Lm*F-02	MS	R	MS	R	R	R

Plant genotypes	Bacterial isolates					
	*Xtg*3	*Xtg*5	*Xtg*8	*Xtg*9	*Xtg*19	*Xtg*29
*Lm*F-03	MS	MS	MS	MS	MS	MS
*Lm*F-04	R	R	R	R	MS	MS
*Lm*G-01	R	MS	MS	MS	R	MS
*Lm*G-02	R [ab]	MS [b]	R [ab]	R [ab]	R [a]	R [ab]
*Lm*G-03	R	MS	MS	MS	MS	MS
*Lm*G-04	R	R	R	R	R	R
*Lm*H-01	R	MS	MS	MS	R	MS
*Lm*H-02	R	MS	R	R	R	R
*Lm*H-03	R	R	R	R	MS	R
*Lm*H-04	R	R	MS	MS	MS	R
*Lm*I-01	R	R	R	R	R	R
*Lm*I-02	R	R	R	R	MS	R
*Lm*I-03	R	R	R	R	R	R
*Lm*I-04	R	MS	S	S	MS	MS
*Lm*J-01	MS	S	S	S	MS	MS
*Lm*J-02	MS	MS	R	MS	R	R
*Lm*J-03	R	R	R	R	R	R
*Lm*J-04	MS	S	S	S	S	S
*Lm*J-05	R	MS	MS	MS	MS	MS
*Lm*K-01	S	S	S	S	S	S
*Lm*K-02	S	S	S	S	S	S
*Lm*K-03	S	S	S	S	S	S
*Lm*K-04	MS	MS	MS	MS	MS	MS
*Lm*K-05	MS	MS	R	MS	MS	MS
*Lm*K-06	MS	MS	S	S	MS	MS
*Lm*L-01	R	MS	MS	MS	MS	MS
*Lm*L-02	R	MS	MS	R	R	MS
*Lm*L-03	R	R	R	R	R	R
*Lm*L-04	R	R	R	MS	R	R
*Lm*L-05	R	MS	R	R	R	R

R: resistant (AUDPC values≤39)

MS: moderately susceptible (39<AUDPC values<60)

S: susceptible (AUDPC values≥60)

2.4.3 SSR genotyping and association analysis

We used EST-derived SSR markers to perform marker-trait associations and to complement the linkage map of the *L. multiflorum* population initially developed by Studer *et al*., (2006; Fig. 2.3). With the 20 SSR primer pairs (Table 2.1), 122 different alleles were detected across the 62 genotypes of *L. multiflorum*. The number of alleles per SSR locus varied from two to 15. Some of these alleles were detected in many different plant genotypes, whereas several alleles were rare and only scored in one or two plant genotypes. AMOVA revealed that 80.55% of the variance in allele frequencies was due to variation between genotypes within

populations, and 19.45% to variation between populations. After LASSO analysis, selected alleles (factors) and the AUDPC values (response) were fitted to a linear regression model followed by ANOVA. Of the 122 scored alleles, 72 different alleles were significantly correlated with resistance to at least one *Xtg* isolate and the three alleles NFA027 (187 bp), NFA027 (193 bp) and G03_013 (193 bp) were significantly correlated with resistance to all six bacterial isolates. Most alleles only explained between 0.001% and 5% of the observed variance for AUDPC values. However, eleven alleles each explained more than 5% of the variance of AUDPC values for resistance to at least one bacterial isolate (Table 2.5).

Table 2.5 Alleles significantly correlated with bacterial wilt resistance, the percentage of the variance for area under the disease progress curve (AUDPC) values they explained, and the linkage group (LG) they mapped to on the *L. multiflorum* reference map (Studer *et al.*, 2006). The alleles listed explained at least 5% of the phenotypic variance by ANOVA with alleles selected with least absolute shrinkage and selection operator (LASSO) at (P<0.001).

Allele	LG	Bacterial isolates					
		Xtg 3	*Xtg* 5	*Xtg* 8	*Xtg* 9	*Xtg* 19	*Xtg* 29
NFA027 (179 bp)	5		8.0%	8.3%	8.2%	8.3%	8.1%
NFA027 (183 bp)	5	12.3%	9.5%		9.5%	9.9%	9.6%
NFA027 (186 bp)	5		12.5%	17.2%	12.4%		
NFA027 (187 bp)	5	34.9%	34.9%	37.4%	34.6%	32.3%	32.4%
NFA027 (193 bp)	5	10.1%	5.9%	5.4%	5.8%	10.3%	10.5%
NFA059 (125 bp)	5						6.0%
G02_037 (197 bp)	1		3.0%	3.3%	3.9%	9.6%	
G03_013 (203 bp)	4				5.8%	9.0%	
G04_034 (162 bp)	4			5.5%			9.3%
G05_121 (226 bp)	4		1.0%	3.4%			8.4%
G05_122 (381 bp)	4	10.7%	0.2%	0.6%	0.6%		0.3%

Five of the alleles explaining more than 5% of the observed variance of AUDPC values were scored on the same NFA027 locus on LG 5. Most notably, NFA027 (187 bp) explained between 32.3% and 37.4% of the total observed variance of AUDPC values across all isolates. Further, one additional allele on LG 5 i.e. NFA059 (125 bp), four alleles on LG 4 i.e. G03_013 (203 bp), G04_034 (162 bp), G05_121 (226 bp) and G05_122 (381 bp), and one allele on LG 1 i.e. G02_037 (197 bp) were observed that explained more than 5% of the observed variance of AUDPC values. Alleles associated with the QTL observed in the initial *L. multiflorum* mapping population were not or only occasionally observed in individuals of *Lm*A, the F_2 progeny of the mapping population, most likely due to the small sample size of only 12 individuals. Across all *L. multiflorum* genotypes used in the present study, none of the

alleles from marker loci that mapped close to the major QTL on LG 4 were significantly correlated with bacterial wilt resistance.

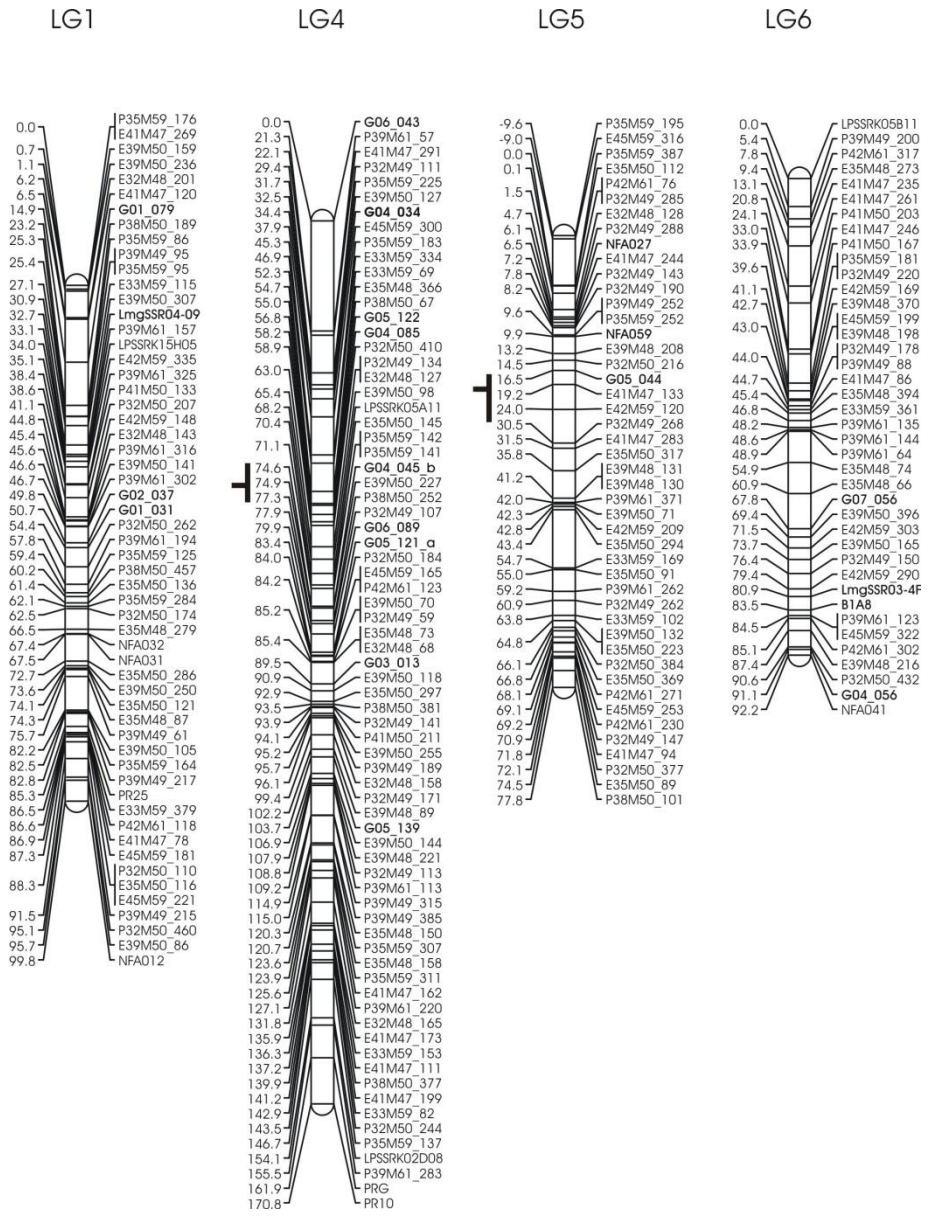

Figure 2.3 Linkage Groups (LGs) 1, 4, 5 and 6 of the genetic linkage map of a *L. multiflorum* reference population (Studer *et al.*, 2006) constructed using 368 amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers complemented with expressed sequence tag (EST)-derived SSR markers (Gxx_xxx). SSR markers in bold were used for marker-trait association analysis (Table 2.1). Scale units are given in centiMorgan (cM). Positions of Quantitative Trait Loci (QTL) for bacterial wilt resistance were re-calculated using multiple QTL model (MQM) mapping based on least square means of resistance from greenhouse data of Studer *et al.*, (2006). The maximum logarithm of the odds (LOD) score position is indicated with a horizontal line, bars represent the interval between two positions obtained at LOD scores two units lower than the maximal score.

2.5 Discussion

A resistance-based strategy for sustainable control of bacterial wilt in forage grasses would strongly benefit from a more thorough understanding of the existence of race-specific resistance mechanisms. This understanding may be especially important for durable disease control, since race-specific resistance usually provides protection against subpopulations of the pathogen only and may be prone to changes in pathogen structure due to genetic changes or migration from other geographic areas. However, the fact that ANOVA revealed no statistically significant interaction of *L. multiflorum* genotypes and *Xtg* isolates and disease severity varied more or less continuously and independently of *Xtg* isolates, suggests that *Xtg* resistance is not conferred by race-specific resistance genes in this species.

These results are congruent with observations from Michel (2001), where the *L. multiflorum* cultivar–bacterial isolate interaction was not significant. However, in the mentioned study, the cultivars used consisted of genetically diverse individuals. Thus, the results do not preclude the existence of plant genotype–bacterial isolate interactions. However, even though we used highly diverse plant genotypes representing a broad range of cultivars and ecotypes, and bacterial isolates representing the broadest range of genetic diversity currently available from Switzerland, we found no evidence for race-specific interactions. Although such results are always limited to the plant genotypes and bacterial isolates used for investigation, the materials used in this study are representative for grassland agriculture in many temperate regions.

The significant differences observed among replications can be explained by varying outside temperatures across the sequentially grown replications and the inability to cool the greenhouse. Growth rates and xanthan production of xanthomonads are affected by temperature and influence disease development (Imaizumi *et al.*, 1999). However, correlation among the four replications was high and significant, confirming the suitability of the experimental approach used.

The ranking in virulence of the six bacterial isolates in the present study was for the most part congruent with the differences in virulence observed by Kölliker *et al.*, (2006) which has been based on three different *L. multiflorum* cultivars. This provides strong support that the bacterial isolates used are reproducibly virulent on different plant material and during different experiments.

Partial resistance to bacterial leaf streak disease was concluded to be plant genotype-dependent in an experiment with wheat genotypes inoculated with *X. translucens* pv. *cerealis* (previously named *X. campestris* pv. *cerealis)* isolates, as no significant genotype–isolate

interaction was detected (El Attari *et al.*, 1996). In contrast, highly significant genotype–*Xanthomonas* isolate interactions have been observed in rice and cassava. Knowledge of these interactions has been crucial for the identification of plant genotypes with specific resistances and pathogen isolates with specific virulence (Nayak *et al.*, 2008; Wydra *et al.*, 2004). However, these two *Xanthomonas* species (*Xoo* infecting rice and *X. axonopodis* pv. *manihotis* (*Xam*) infecting cassava) possess a very high level of genetic diversity (60-90%) indicating that they underwent substantial genetic differentiation (Hua *et al.*, 2007; Restrepo *et al.*, 2000). On the other hand, *Xtg* isolates have been shown to possess a high level of genetic similarity (>86%) based on amplified fragment length polymorphism (AFLP) markers and low genetic diversity (Kölliker *et al.*, 2006). Further, it has been shown that pathogen variation is suppressed by mixtures of genetically diverse host plants (Zhu *et al.*, 2000). This has been shown for rice cultivars grown in monocultures compared to mixtures of different rice varieties and the rice blast causing pathogen *Magnaporthe grisea*. Low genetic diversity of *Xtg* and genetically diverse host plant populations therefore may represent an impediment to adaptation. That is, the evolutionary potential of *Xtg* during the interaction with its host may be limited and the frequency of occurrence of race-specific interactions may also be reduced. Cultivars of forage grasses are usually composed of numerous individual genotypes containing several different resistance genes and they are often not grown in pure stands but in mixtures with other grasses and legume species. Therefore, selection pressure may be too low for the development of race-specific resistance in the *L. multiflorum–Xtg* interaction. Qualitative race-specific resistance has been shown to exist in *L. multiflorum* against other pathogens such as *Puccinia coronata* (Schubiger *et al.*, 2006). Therefore, not only the genetic composition of the host, but also characteristics of the pathogen such as the evolutionary potential may be responsible for the absence of race-specific interactions.

Based on the insignificant *L. multiflorum–Xtg* interaction, one might expect of the marker-trait associations that all alleles would correlate with bacterial wilt resistance across all isolates. This was true for one allele of the NFA027 locus (187 bp) which mapped to LG 5 and explained up to 37.4% of the total observed variance of AUDPC values across all isolates. This is a further indication of major broad spectrum resistance not limited to specific isolates. The major QTL on LG 4 described by Studer et al., (2006) could not be confirmed in the F_2 progeny derived from the initial mapping population due to the small number of parental alleles observed in the investigated individuals. However, our intention was rather to investigate whether such a QTL was present in more distantly related germplasm. The markers that mapped close to the major QTL on LG 4 (Fig. 2.3) observed by Studer *et al.*,

(2006) did not have major effects on the total observed variance of AUDPC values across all genotypes, indicating the existence of additional resistance genes or QTL. Four alleles that mapped to LG 4, but not in vicinity of the major QTL, explained up to 10.7% of the total observed variance of AUDPC values, suggesting that there may be other genes with minor contributions to *Xtg* resistance. In addition, the alleles that explained between 5.5% and 9.3% of variance of AUDPC values across only one or two bacterial isolates may be an indication for the existence of several minor race-specific resistance genes with only small effects.

In summary, we conclude from our greenhouse experiment and the marker-trait associations that in a wide range of *L. multiflorum* genotypes, bacterial wilt resistance is effective against a broad range of *Xtg* isolates with different virulence. Consequently, no major race-specific resistance seems to exist in the *Xtg–L. multiflorum* interaction. Therefore, resistance breeding may be based on one or a few *Xtg* isolates in order to select for *L. multiflorum* genotypes with high levels of *Xtg* resistance without compromising durability of resistance.

2.6 Acknowledgements

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3 Transcriptional responses of Italian ryegrass during interaction with *Xanthomonas translucens* pv. *graminis* reveal novel candidate genes for bacterial wilt resistance

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3.1 Abstract

Xanthomonas translucens pv. *graminis* (*Xtg*) causes bacterial wilt, a severe disease of forage grasses such as Italian ryegrass (*Lolium multiflorum* Lam.). In order to gain a more detailed understanding of the genetic control of resistance mechanisms and to provide prerequisites for marker assisted selection, the partial transcriptomes of two Italian ryegrass genotypes, one resistant, and one susceptible to bacterial wilt were compared at four time points after *Xtg* infection. A cDNA microarray developed from a perennial ryegrass (*L. perenne*) expressed sequence tag (EST) set consisting of 9,990 unique genes was used for transcriptome analysis in Italian ryegrass. An average of 4,487 (45%) of the perennial ryegrass sequences spotted on the cDNA microarray were detected by cross-hybridization to Italian ryegrass. Transcriptome analyses of the resistant vs. the susceptible genotype revealed substantial gene expression differences (>1200) indicating that great gene expression differences between different Italian ryegrass genotypes exist which potentially contribute to the observed phenotypic divergence in *Xtg* resistance between the two genotypes. In the resistant genotype, several genes differentially expressed after *Xtg* inoculation were identified which revealed similarities to transcriptional changes triggered by pathogen associated molecular patterns (PAMPs) in other plant-pathogen interactions. These genes represent candidate genes of particular interest for the development of tools for marker assisted resistance breeding.

3.2 Introduction

Bacterial wilt is a major disease in various forage grasses including Italian ryegrass (*Lolium multiflorum* Lam.), causing substantial losses in forage crop production and infestation of important breeding material. The disease is caused by the bacterial pathogen *Xanthomonas translucens* pv. *graminis* (*Xtg*; Egli *et al.*, 1975) Vauterin et. al. (1995), which occludes the xylem vessels, and causes wilting symptoms and necrosis of the leaves. Susceptible plants may die within only a few days after infection. Breeding for resistant cultivars is a major objective and has lead to development of cultivars with increased resistance to bacterial wilt. Nevertheless, despite intensive breeding efforts, no complete resistance has been achieved to date, and highly susceptible individuals still occur in advanced breeding populations (Michel, 2001). This may be due to the out-breeding reproductive mode of this species and the population-based breeding schemes often used to improve ryegrasses. In such complex interactions, a detailed understanding of the genetic control of resistance mechanisms is crucial for further improvements in *Xtg* resistance breeding.

Transcriptome and quantitative trait loci (QTL) analyses represent powerful tools for elucidating host defence responses and for the identification of genetic markers linked to disease resistance. QTL mapping in a *L. multiflorum* mapping population identified one major QTL on linkage group (LG) 4 associated with bacterial wilt resistance explaining up to 84% of the total phenotypic variance (Studer *et al.*, 2006). This major QTL indicated the presence of major qualitative resistance. However, phenotypic and molecular genetic evaluation of race-specific interactions between different *Xtg* isolates and *L. multiflorum* genotypes revealed no indication of major qualitative *Xtg* resistance (Wichmann *et al.*, 2011). Thus, although major QTL may be found in particular germplasm, additional unknown genes or QTL control the quantitative *Xtg* resistance in *L. multiflorum*. The analysis of transcripts differentially expressed as a response to infection may allow further elucidation of the genetic control of host resistance. Preliminary transcriptome analyses of a resistant *L. multiflorum* genotype inoculated with *Xtg* using cDNA-amplified fragment length polymorphism (AFLP) identified a number of differentially expressed transcript derived fragments (TDF) at different time points after inoculation (Rechsteiner *et al.*, 2006) and therefore demonstrated the suitability of the approach. However, the small number and the short lengths of the TDF analyzed limited the utility and generality of the results for further, candidate gene based investigations of the genetic control of *Xtg* resistance. In addition, the cDNA-AFLP analysis focused only on one single *L. multiflorum* genotype not taking into account the high genetic diversity of individual genotypes present in populations and cultivars due to the out-breeding reproduction system of the species. Therefore, a more comprehensive transcriptome analysis using more than one genotype and a larger number of transcripts is needed to gain a more detailed insight into genes and pathways involved in defence responses against *Xtg*.

Microarray-based transcriptome analyses allow for the simultaneous detection of modulated expression of several thousand transcripts and are therefore suitable for large scale analyses. In crops such as rice, cassava, citrus or tomato, microarray analyses of interactions with *Xanthomonas* spp. have revealed differential expression of specific genes encoding cell-wall modifying proteins, protein kinases involved in signalling pathways and genes triggered by pathogen-associated molecular patterns (PAMPs; Cernadas *et al.*, 2008; Gibly *et al.*, 2004; Li *et al.*, 2006b). For example, a number of genes are co-regulated during the interactions of rice with *X. oryzae* pv. *oryzae* (*Xoo*) and with *Magnaporthe grisea* (the fungal pathogen causing rice blast) indicating shared defence pathways (Li *et al.*, 2006b). In addition, a number of candidate genes involved in signal transduction co-locating with a major QTL for broad-spectrum *Xoo* resistance and a QTL for submergence tolerance on chromosome 5 have

been identified in rice, indicating that submergence tolerance and broad-spectrum *Xoo* resistance share a common signalling system (Kottapalli *et al.*, 2007). In citrus, many genes associated with PAMP recognition were commonly modulated after *X. axonopodis* pv. *aurantifolii* and *X. axonopodis* pv. *citrii* infection (Cernadas *et al.*, 2008). Although *L. multiflorum* as well as *Xtg* share phylogenetic similarities with some of the host and pathogen species described above, the *L. multiflorum* x *Xtg* interaction is unique in many respects. The pathogen has a relatively broad host range (Egli & Schmidt, 1982), host resistance is characterized by partial rather than complete resistance and race-specific interactions have not, so far, been observed (Wichmann *et al.*, 2011). In addition, *L. multiflorum* populations and cultivars are characterized by a high genetic diversity, while *Xtg* isolates have been shown to share high genetic similarity (Kölliker *et al.*, 2006). A detailed transcriptome analysis of this host x pathogen interaction is therefore crucial for a more profound understanding of resistance mechanisms and for developing tools for marker assisted resistance breeding. However, to date, there is no representative microarray available for *L. multiflorum* and sequence information available is limited. In such cases, cross-species hybridization (CSH) using microarrays with sequences from related species represents a valuable tool for transcriptome analyses (Bar-Or *et al.*, 2007). Due to the close phylogenetic relationship of *L. multiflorum* and *L. perenne* (Catalan *et al.*, 2004), the 9,365 unique EST sequences developed at Det Jordbrugvidenskabelige Fakultet (DJF), Aarhus University (Asp *et al.*, 2007) represents a promising resource for transcriptome analysis in *L. multiflorum*.

The first aim of this study was to develop a cDNA microarray using a unique EST sequence set of *L. perenne* and to evaluate this cDNA microarray for transcriptome analyses in *L. multiflorum.* The second aim was to compare the transcriptomes of an inoculated and control-treated *L. multiflorum* genotype with a high level of resistance to bacterial wilt in order to identify candidate resistance genes for marker assisted selection. The third aim was to compare the transcriptomes of a resistant and a susceptible *L. multiflorum* genotype in order to elucidate genotypic differences contributing to varying levels of resistance to bacterial wilt.

3.3 Materials and methods

3.3.1 Construction of a *Lolium perenne* cDNA microarray

The set of unique genes for construction of the cDNA microarray was identified by clustering and assembly of 25,744 high-quality *Lolium perenne* EST sequences. The 25,744 ESTs were assembled into 3,195 tentative consensus sequences and 6,170 singletons, thus representing 9,365 unique sequences (Asp *et al.*, 2007). In addition, a total of 625 expressed sequences of

Lolium either retrieved from public databases (http://ncbi.nlm.nih.gov), or representing candidate genes for vernalization (Andersen *et al.*, 2006), for *Xtg* resistance identified by cDNA-AFLP (Rechsteiner *et al.*, 2006), laccases (Schejbel *et al.*, 2008), and resistance gene analogues (RGA; Ikeda, 2005) were included resulting in a total of 9,990 unique genes. Plasmid DNA of the unique genes was prepared from all clones by MWG Biotech AG (Ebersberg, Germany). The cDNA inserts were amplified by PCR using standard M13 forward and reverse primers, Taq DNA polymerase (0.8 U; Fermentas, Vilnius, Lithuania) in reaction volumes of 100 µl containing reaction buffer (10 mM Tris, pH 8.8; 50 mM KCl; 0.08% Nonidet P40; 0.5 mM MgCl2; 0.3 mM (each) dNTP). The PCR reactions were performed in a MJ Research PTC-225 Thermal Cycler (MJ Research, Waltham, MA, USA) and the cycling conditions were 3 min at 95˚C, followed by 35 cycles of 30 s at 95˚C, 30 s at 61˚C, 3 min at 72˚C, and a final extension step of 10 min at 72°C. PCR products were precipitated at -20˚C for 12 h with 3 (v/v) 96% ethanol and 1/10 (v/v) 3 M sodium acetate, pH 5.2, and subsequently centrifuged at 4000 rpm for 1 h in an Eppendorf 5810R centrifuge (Eppendorf AG, Hamburg, Germany). The DNA pellet was air-dried and resuspended in 20 µl of water. The quality of the PCR products was visually inspected by agarose gel electrophoresis. A total of 10 µl (200–500 ng) of each sample was vacuum-dried and resuspended in 5 µl of 50% dimethyl sulphoxide (DMSO; Sigma-Aldrich, St. Louis, MO). The samples were spotted onto Nexterion Slide A (Schott Nexterion, Jena, Germany) using a QArrayMini spotter (Genetix, New Milton, UK). Following spotting, the microarrays were UV crosslinked at 250 mJ in a UV Stratalinker 1800 (Stratagene, Santa Clara, CA, USA) and kept dry in the dark at room temperature until hybridization.

3.3.2 Bacterial isolates and plant material

The *L. multiflorum* genotypes *Lm*B-01 (partially resistant to *Xtg*) and *Lm*K-01 (highly susceptible to *Xtg*) previously characterized for *Xtg* resistance (Wichmann *et al.*, 2011) were used for transcriptome analyses. Genotype *Lm*B-01 originated from a Syn1 progeny of a polycross with nine elite genotypes from Agroscope Reckenholz-Tänikon, and genotype *Lm*K-01 is an individual selected from the commercially available cultivar Adret (Verneuil Recherche, Verneuil-Etang, France). Both *L. multiflorum* genotypes were clonally propagated and *Xtg* inoculation and control treatment were performed using the leaf clipping method as described in Kölliker et al. (2006). Plants were arranged in a split-plot design with the sampling time point as block factor and four biological replicates per treatment and genotype, resulting in 16 clones per genotype. The *Xanthomonas translucens* pv. *graminis* isolate *Xtg*29 (Kölliker *et al.*, 2006), which was stored at -70ºC in GYC (glucose 2% (w/v), yeast extract

1% (w/v), $CaCO_3$ 2% (w/v)) broth containing 15% (v/v) glycerol, was used for inoculation. For the control treatment, plants were cut with sterile scissors without inoculum. Plant leaves and sheaths were cut at 2 cm above soil and the total harvest was used for total RNA extraction at four time points post inoculation or control treatment: 8 hours, 48 hours, 192 hours and 288 hours post inoculation (hpi) or hours post control treatment (hpc). Early disease symptoms such as wilting of the tips of the leaves were only observed on the plants of the susceptible genotype sampled at the last time point after infection at 288 hpi (data not shown). Dye swaps were included into the experimental design with two biological replicates assigned to each labelling dye (Fig. 3.1). Direct comparisons between the control-treated susceptible and the inoculated susceptible plants were not included into the experimental design since the main interest was laid on the comparison of non-inoculated vs. inoculated plants of the resistant genotype and the comparison of the two genotypes under the two different treatments. However, effects of the indirect comparisons can still be estimated. All tissue samples were immediately frozen in liquid nitrogen and stored at -70ºC prior to total RNA extraction.

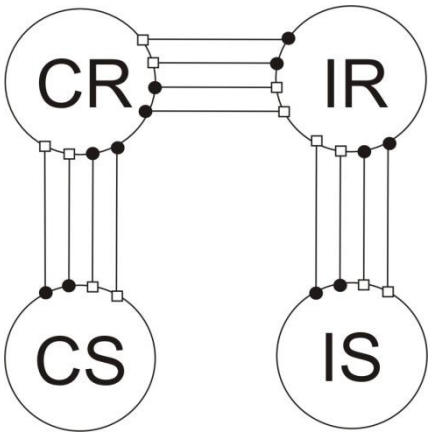

Figure 3.1 Experimental design of microarray analyses performed for each sampling time point. Circles represent labelled mRNA samples with the following genotypes and treatments: C= control treatment, I = inoculated with *Xanthomonas translucens* pv. *graminis*, R = resistant *Lolium multiflorum* genotype, S = susceptible *L. multiflorum* genotype. Lines indicate mRNA samples hybridized to the same microarray. The samples containing black circles were labelled with Cy3 and the samples containing a white box were labelled with Cy5.

3.3.3 RNA extraction and reverse transcription

The plant leaves were ground in liquid nitrogen and 300 mg of plant material per plant sample was used for total RNA isolation using the FastRNA Pro Green Kit® (MP Biomedicals, Irvine, CA, USA) and FastPrep® Instrument (MP Biomedicals). The FastPrep® setting 6.0 was used for 40 seconds. Thereafter, RNA extraction and reverse transcription were

performed as described by Gregersen et al. (2005). The amino-allyl cDNA concentration was measured using the Synergy2 plate reader (BioTek, Winooski, VT, USA) measuring the absorbance at 260 nm. After cDNA quantification, the samples were dried in a Vacufuge (Eppendorf) and resuspended in 10 µl of 0.1 M sodium bicarbonate pH 9.0. Labelling of second strand cDNA was performed with the CyDye (Cy3/Cy5) Post-Labelling Reactive Dye Pack (Amersham Pharmacia, UK) in the dark for 2 hours at 35ºC. After labelling, the samples were purified using the Qiaquick PCR purification kit and PB buffer (Qiagen, Hilden, Germany). Quantification of labelled cDNA and incorporated CyDye was carried out with a spectrophotometer measuring the absorbance at 260 nm and 550 nm for Cy3, and 260 nm and 650 nm for Cy5, respectively.

3.3.4 Hybridization, washing and scanning of spotted microarrays

The target cDNA was prepared from approximately 25 pmol of each the Cy3- and Cy5-labeled cDNA sample, dried with the Vacufuge (Eppendorf) and resuspended in 5 µl of sterile water and 45 µl of Nexterion® Hyb buffer (Schott Nexterion). Before hybridization, the spotted microarrays were blocked in 5 g of succinic anhydride, 280 ml of 1-methyl-2-pyrrolidinone and 12.5 ml of sodium borate (1 M, pH 8) for 15 minutes. After blocking, the microarrays were immersed into 0.1% (w/v) SDS and water for 20 seconds followed by a denaturation of 3 minutes in boiling water and spin-drying in at 1500 rpm for 8 minutes.

Before hybridization, the target cDNA was denatured at 95ºC for 3 minutes and cooled for 30 seconds on ice. The target cDNA was pipetted on the middle of the spotted area of the microarray and covered carefully with a LifterSlip (Eerie Scientific Company, Portsmouth, NH, USA). The microarray was placed in a hybridization chamber containing 1xSSC and incubated at 65ºC for 16 h. The LifterSlip was removed by gentle agitation in 1xSSC and 0.2% SDS. Then the microarrays were washed once in pre-warmed 1xSSC and 0.2% SDS for 10 min, twice in pre-warmed 0.2xSSC and 0.1% SDS and twice at room temperature in 0.1xSSC for 1 minute. After washing, the microarrays were again spin-dried and scanned. Scanning was performed with a GenePix® Personal 4100A (Axon Instruments, Union, CA, USA) microarray scanner and the PMT gains (exposure settings) were optimized individually for each microarray.

3.3.5 Microarray data analyses and statistics

Quantification of hybridization signals was performed using the GenePix® Pro 6.0 software (Axon Instruments) aligning the spot grids for each spot automatically with manual adjustments. The R software 2.8 and the LIMMA package (Smyth, 2005) were used to

normalize the microarray data. Normalizations within arrays were performed in a signal dependent manner using the LOWESS (locally weighted linear regression) method to remove intensity-dependent variation in dye bias by applying a smoothing adjustment that removes such variation (Yang & Speed, 2002). This was followed by between array normalizations using the quantile method as proposed by Bolstad et al. (2003). Diagnostic plots were created using "maQualityPlots" function of the arrayQuality package (Paquet & Yang, 2008). Data producing unsatisfactory diagnostic plots were discarded. Generation of lists of differentially expressed genes was performed by means of the moderated t-statistics (Lonnstedt & Speed, 2002) using a *p* value threshold of $p<0.01$. For the comparison between genotypes, which otherwise would have yielded excessive numbers of differentially expressed genes, a $\log_2$ fold change (FC) threshold of 0.8 was used additionally. Since subtraction of background signals increase spot variation (Qin & Kerr, 2004), foreground signal alone was used for normalization and analysis of differential gene expression. DNA sequences and predicted protein sequences were analyzed and annotated using GenBank and the blastx database (http://ncbi.nlm.nih.gov/BLAST/) considering annotations with E-values<1E-06. Assignment of the genes to functional categories was performed following the description of the genes in public databases according to functional categories in the GO database (Ashburner *et al.*, 2000).

3.3.6 Quantitative real-time PCR

Differential expression of genes observed with the microarray assay was verified by quantitative real-time PCR using an iCycler (Bio-Rad, Hercules, CA, USA) and the iQ™ SYBR® Green Supermix (Bio-Rad). The plant material from the four replicates of each treatment and time point sampled for the microarray experiment was pooled for total RNA extraction as described above. Before cDNA synthesis, total RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen) including DNase treatment (Qiagen). cDNA synthesis was performed in a 60 µl volume from 3 µg of total RNA using an oligodT_{25}-primer (Microsynth, Balgach, Switzerland) and Superscript II (Invitrogen, Carlsbad, CA, USA) according to the manufacturers recommendations. The primer pairs for a number of selected genes potentially involved in *Xtg* resistance or with a high FC value were designed with the Primer3 tool (Rozen & Skaletsky, 2000) to amplify fragments of approximately 150-250 bp and to be of similar GC content and melting temperature (Table 3.1).

Table 3.1 Sequence IDs, forward and reverse primer sequences and annotations of the genes confirmed by quantitative real-time PCR. The comparison in which the genes were detected to be differentially expressed is indicated: C= control treatment, I = inoculated with *Xanthomonas translucens* pv. *graminis*, R = resistant *Lolium multiflorum* genotype, S = susceptible *L. multiflorum* genotype.

Sequence ID[1]	Forward primer (5'→3')	Reverse primer (5'→3')	Comparison	Annotation[2]
r_010d_c02	AGCAAACCTCGACAAGCCTA	AGCACCGTGAGGATCTCTGT	CR↔IR	Germin-like protein (GLP6)
r_010d_c04	TTCAGGTCCCGTTCTACTGG	CACCGCTCTGTTGTCTGTTG	CR↔IR	Low silicon protein (Lsi1)
rg6_008b_d08	TCATCGCCCTCATCCTTATC	GGGCCAGAGCACACTAAGAG	CR↔IR	Synaptobrevin-like protein
gsa_007d_f02	GGTTTTCTTCCCATTTGGACC	CGAGGTAAAGCTCAACAGACG	CR↔IR	TMBIM4
ve_007b_e10	CATGGTTGGTTGGTTCTGTG	TGCCTCAAGAACAGCAACAG	CR↔IR	Structural protein MFS18
gsa_007c_h09	GGGACAAGGAGAAGATGCTG	CAAACAGGCCACGGTTATTT	CR↔IR	Ankyrin
sb_001a_f10	GTGTATGTATGGATGTGTGTGTG	TGGTCTCATTCATCGCAAGA	CR↔IR	Unknown
rg3_011d_b12	GACATACGTGGTGCAGGATG	GGTAGCTACAGCCTCCTCGT	CR↔IR	Unknown
rg3_011a_h07	CGATACCTTTCCCGACATTG	GCCAAGGGATAAATCGAGGA	CR↔IR	Unknown
r_003d_g12	TGCCAGAGCTTCGTGAATAA	CGTAGCTTCCCAAGACATGC	CR↔CS	Unknown
rg1_014c_b10	CATGTGCCAGCTCTGACCTA	CCAAGGTATTCGATGCCACT	CR↔CS	Leucine Rich Repeat
Lac_11	CACCAAGAGCATCGTGACAG	CCGGTGATGGTGAAGTTGTA	CR↔CS	Laccase 11
rg1_011a_g05	CGCTCTGGACCCTAACAGTC	GCATTCATCAAAGTCGAGCA	CR↔CS	Wheat induced resistance (WIR 1)
rg1_015d_f09	ATCCCTCAAGGCTTCCAGAT	AGTTTCCTCACGGCAATCAC	CR↔CS	Serine/threonine protein kinase
sb_003c_e08	GGCAGTACGGGAGGATTACA	TCTGTACTGTTCGGCTGTGG	CR↔CS	Peroxin 14
sb_007b_a08	TGCGTGGATTACTACGACCA	GGGAAGGTATTCAGCAGCAG	CR↔CS	Peroxidase 53
eIF-4a	GGTCGTGTGTTTGACATGCT	CCTTGAAACCACGAGAAAGC	all	Eukaryotic Initiation factor 4a
eEF-1α	GGCTGATTGTGCTGTGCTTA	CTCACTCCAAGGGTGAAAGC	all	Eukaryotic elongation factor 1α

[1] unique identifier. Contains information about the cDNA library (Asp et al. 2007) that the sequence originated from.
[2] derived from GenBank and the blastx database (http://ncbi.nlm.nih.gov/BLAST/)

The specificity of primer pairs was verified by melting curve analysis. The eukaryotic initiation factor 4a (eIF-4a) and the eukaryotic elongation factor 1α (eEF-1α) were used as internal reference genes, since they were shown to be most suitable for mRNA quantification due to stable expression in different tissues and under different conditions in *L. perenne* (Martin *et al.*, 2008). PCR amplifications were performed using primers described by Martin et al. (2008) in 25 µl volumes using 2 µl of 1:10 fold diluted cDNA. Thermal-cycling conditions were as follows: initial denaturation step at 95ºC for 3 minutes, 45 cycles of 30 sec at 95ºC, 30 sec at 61.4ºC, 30 sec at 72ºC, final extension at 72ºC for 5 min. Expression ratios of three technical replicates were averaged for each sample. Quantification of the relative changes in gene expression was performed using the Pfaffl method and the REST software (Pfaffl *et al.*, 2002).

3.4 Results

3.4.1 Overview of global gene expression

An average of 4,487±196 ESTs (45%) of the 9'990 *L. perenne* EST sequences spotted on the cDNA microarray were detected inboth channels with a signal/background ratio >1.5 by target cDNA from *L. multiflorum* across all hybridizations included in the analysis. In addition, the average background intensity value across all hybridizations for both channels was at 294.4 ±19.8.

3.4.2 Transcriptional changes following *Xtg* inoculation in the resistant genotype

Comparisons of control-treated and *Xtg* inoculated plants were performed with the partially resistant *L. multiflorum* genotype *Lm*B-01. The transcriptome analysis of the resistant genotype revealed in total 158 genes differentially expressed after *Xtg* inoculation (Fig. 3.2, Supplementary Table 1). Twenty up-regulated genes were observed at 48 hpi, 52 genes were differentially expressed 192 hpi (42 up- and 10 down-regulated), and 124 genes were differentially expressed 288 hpi (76 up- and 48 down-regulated). No genes revealed significant differential expression 8 hpi. Of the 158 differentially expressed genes in total, 33 genes (21%) were differentially expressed at more than one time point after inoculation (Fig. 3.2, Supplementary Table 1). For example, 5 genes were up-regulated at the three sampling time points 48, 192 and 288 hpi, 2 were up-regulated at 48 and 192 hpi, 6 were up-regulated both 48 and 288 hpi and 20 were up-regulated 192 and 288 hpi.

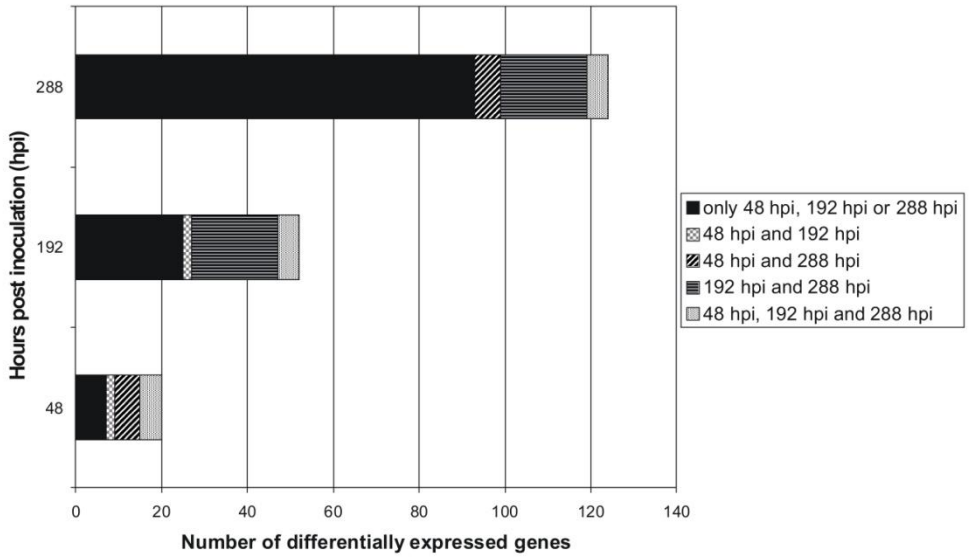

Figure 3.2 Number of genes differentially expressed in the partially resistant *Lolium multiflorum* genotype *Lm*B-01 after *Xanthomonas translucens* pv. *graminis* (*Xtg*) inoculation at three time points post inoculation (48, 192 and 288 hpi). Identical filling patterns indicate genes differentially expressed at multiple time points.

Defence and stress related genes

Defence related genes that were up-regulated after *Xtg* inoculation included a gene encoding the germin-like protein 6 (GLP6; r_010d_c02), the Verticillium wilt disease resistance protein (Ve2; rg3_008d_a09), the precursor of the pathogenesis-related protein 5 (csAtPR5; r_008d_h09), the nonspecific lipid-transfer protein 2 (sb_004a_g05), and the transmembrane BAX-inhibitor motif containing protein 4 (TMBIM4; gsa_007d_f02). Down-regulated defence related genes included genes encoding the laccase 11 (LAC11) and the pathogenesis-related protein 4 (PR4; rg5_007a_c11). The stress-related gene encoding the Low silicon protein 1 (Lsi1; r_010d_c04) was also up-regulated after *Xtg* inoculation.

Genes involved in signal transduction

Genes involved in signal transduction that were up-regulated after *Xtg* inoculation included genes encoding a victorin binding protein (ve_005b_b02), an ankyrin (gsa_007c_h09), the Pto kinase interactor 1 (p_001c_b08), a leucine rich repeat protein (rg2_004_f05), an annexin (r_004d_e11) and the brassinosteroid-insensitive 1 (r_014a_c01) protein, and a signal peptidase containing a 18K chain (rg6_008c_g08). A gene encoding a signal recognition receptor (sb_005a_a05) was down-regulated after *Xtg* inoculation.

Other genes

Two genes encoding glutathione transferases (r_003c_g07 and rg1_008a_h04) responsible for detoxification and a gene encoding a synaptobrevin-like vesicle associated membrane protein (rg6_008b_d08) were up-regulated after *Xtg* inoculation.

3.4.3 Transcriptional differences between the resistant and the susceptible genotype

Comparisons of expression profiles of the resistant genotype and the susceptible genotype across all sampling time points revealed a total of 1,203 differentially expressed genes (Fig. 3; Supplementary Tables 2 and 3). Thereof, 624 genes were up-regulated in the resistant genotype and 579 genes were up-regulated in the susceptible genotype. Of the 624 genes up-regulated in the resistant genotype, 38 (6.1%) genes were only detected after control treatment, 525 (84.1%) were only detected after *Xtg* inoculation, and 61 (9.8%) were detected both after control treatment and after *Xtg* inoculation (Fig. 3.3).

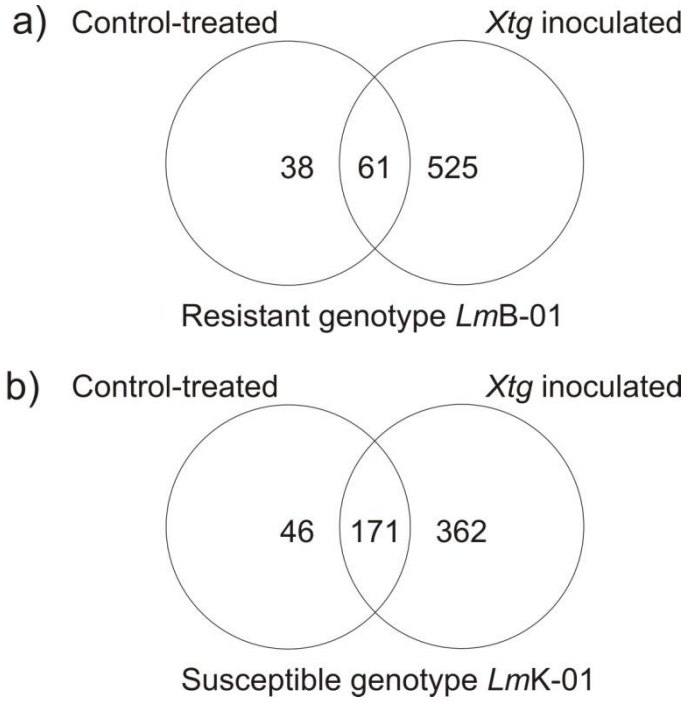

Figure 3.3 Venn diagram of the genes from all time points up-regulated in the resistant *Lolium multiflorum* genotype *Lm*B-01 when compared to the susceptible genotype *Lm*K-01 (a) and in the susceptible genotype *Lm*K-01 when compared to the resistant genotype *Lm*B-01 (b) after control treatment and inoculation with *Xanthomonas translucens* pv. *graminis* (*Xtg*).

Of the genes up-regulated in the resistant genotype, 426 (68%) showed no sequence homology to genes deposited in public databases or were homologous to plant genes with unknown functions and hypothetical proteins. Of the 579 genes up-regulated in the

susceptible genotype, 46 (7.9%) genes were only detected after control treatment, 362 (62.5%) genes were only detected after *Xtg* inoculation, and 171 (29.5%) genes were detected after control treatment and after *Xtg* inoculation. The number of genes with average FC between 0.8 and 1 after *Xtg* infection was comparable between the resistant and the susceptible genotype (Fig. 3.4).

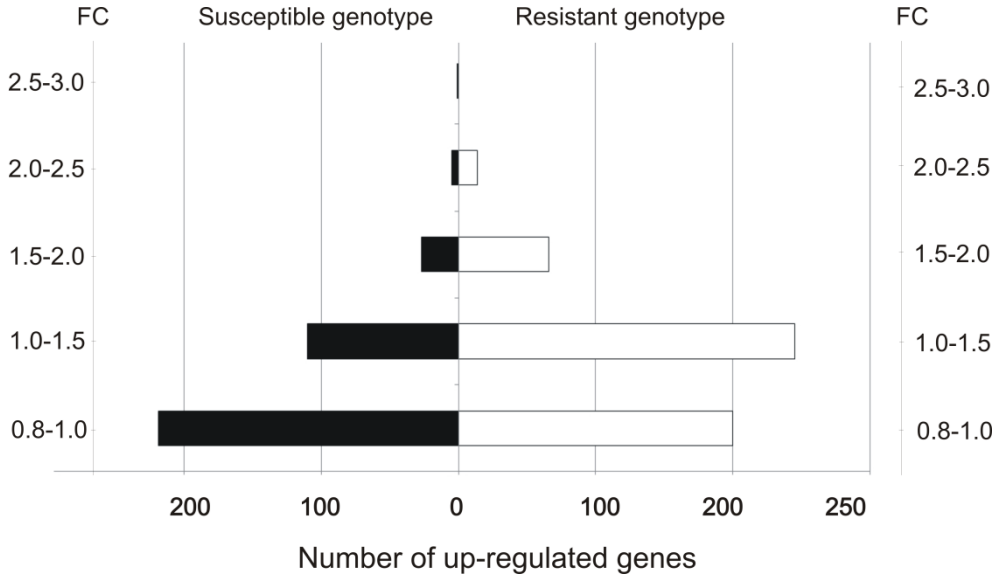

Figure 3.4 Histogram of the fold change (FC) of the numbers of genes from all sampling time points up-regulated in the susceptible (black; n=362) and the resistant (white; n=525) genotype after *Xanthomonas translucens* pv. *graminis* infection**.**

On the other hand the numbers of genes with FC values between 1 and 1.5 and 1.5 and 2 were twofold higher in the resistant genotype when compared to the susceptible genotype. At the four sampling time points (8, 48, 192 and 288 hours post control treatment; hpc), 25, 34, 34 and 29 genes were up-regulated in the resistant genotype and 105, 119, 86 and 53 genes in the susceptible genotype, respectively (Fig. 3.5). Through comparison of the transcriptomes of the two genotypes after *Xtg* inoculation, 112, 125, 452 and 34 genes were up-regulated in the susceptible and 18, 483, 318 and 51 in the resistant genotype 8, 48, 192 and 288 hpi.

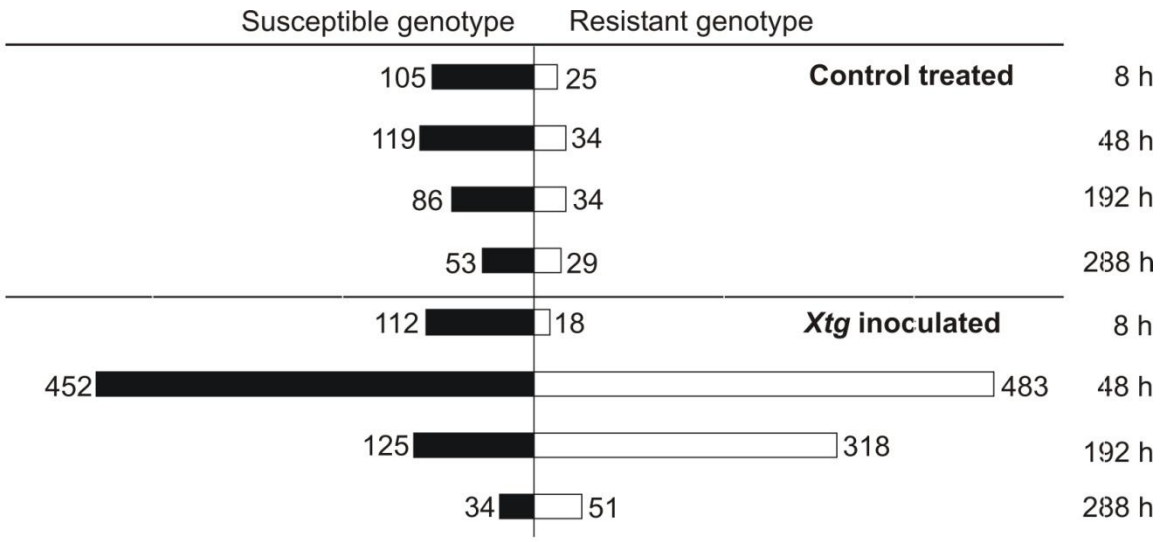

Figure 3.5 Number of up-regulated genes in the susceptible *Lolium multiflorum* genotype *Lm*K-01 (black) and in the partially resistant genotype *Lm*B-01 (white) 8, 48, 192 and 288 hours after control treatment and *Xtg* inoculation. A number of genes were simultaneously up-regulated at more than one time point or both after control treatment and after *Xtg* inoculation.

Of the 579 genes up-regulated in the susceptible genotype, 370 (64%) genes showed no similarities to other plant genes or were homologous to genes with unknown functions and hypothetical proteins (E-value<1.00E-06). Functional categorization was performed as described above and genes with ontologies related to defence and stress are described in more detail.

Defence and stress-related genes up-regulated in the resistant genotype

Defence related genes up-regulated in the resistant genotype included genes encoding a BAX inhibitor 1 (rg6_008a_f08), a 23 kDa jasmonate-induced protein (rg3_005a_h01), a homeobox-like resistance protein (rg3_012a_e02), LAC11, resistance gene analog 7 (RGA7; rg1_006b_g04), the wheat induced resistance protein 1 (WIR1; rg1_011a_g05), avrRpt2-induced protein 2 (AIG2; rg1_013a_d11). Stress-related genes included genes encoding two heat shock proteins (gsa_001b_f05 and r_009c_h12), the universal stress protein (rg3_009a_b11), the chaperonins (rg3_009d_e09 and ve_004a_e03) and the salt tolerance protein 5 (rg3_011a_d10).

Defence and stress related genes up-regulated in the susceptible genotype

Defence related genes up-regulated in the susceptible genotype included genes encoding the powdery mildew resistance protein MLA6 (r_007d_d10), two different lipid transfer proteins (LTP; gsa_007d_e04; sb_001b_b03) and the erwinia induced protein 2 (r_007d_d06). The

genes encoding stress-related proteins included genes encoding a ferredoxin (rg6_014b_f03), a wound-induced protease inhibitor (r_004a_g09), a HVA22-like protein (r_006b_g06), the low molecular mass heat shock protein Oshsp17.3 (r_007d_d03), an early-responsive to dehydration protein (r_009a_c10), the 17.8 kDa class II heat shock protein (r_010c_b08), the cytosolic chaperonin delta-subunit (r_012b_b08) and the chloroplast heat shock protein 70 (ve_006a_c02).

Table 3.2 — Functional categorization of genes differentially expressed between the partially resistant and the susceptible *Lolium multiflorum* genotypes after control treatment (C) and inoculation with *Xanthomonas translucens* pv. *graminis* (I) according to the GO database (Ashburner et al. 2000).

	Resistant genotype			Susceptible genotype		
Functional category	C	I	C and I	C	I	C and I
Amino acid and carboxylic acid metabolism	2	7	2	0	5	6
Autophagy	0	2	0	0	0	0
Carbohydrate metabolism	1	17	1	0	9	4
Cell-wall modification	0	2	0	0	1	1
Defence related	2	9	2	0	3	2
Detoxification	0	2	0	0	2	3
Hormone pathway	0	4	0	0	2	4
Lipid metabolism	0	8	1	1	5	1
Oxidative burst	1	3	3	0	2	1
Phosphorylation/ dephosphorylation	1	11	0	0	10	2
Secondary metabolism	0	1	1	0	0	2
Signal transduction	2	7	0	1	7	2
Stress related	0	6	0	1	7	1
Transcription factor	1	14	0	2	9	3
Transport	0	5	2	1	6	1
Ubiquitination	0	5	1	0	3	4
Uncategorized	7	60	7	10	61	29
Unknown function	21	362	41	30	230	105
Total	38	525	61	46	362	171

3.4.4 Confirmation of differential expression

Nine of the genes differentially expressed in the resistant genotype after *Xtg* inoculation were analyzed using quantitative real-time PCR. Up-regulation was confirmed for the six genes with known functions (Fig. 3.6) as well as for genes with the sequence IDs rg3_011a_h07, rg3_011d_b12 and sb_001a_f10, which did not reveal any sequence homology to sequences deposited in public databases. In addition, real-time quantitative PCR confirmed that the genes with the IDs: r_003d_g12, rg1_014c_b10, Lac_11, rg1_011a_g05, rg1_015d_f09,

sb_003c_e08 and sb_007b_a08 were up-regulated in the resistant genotype when compared to the susceptible genotype.

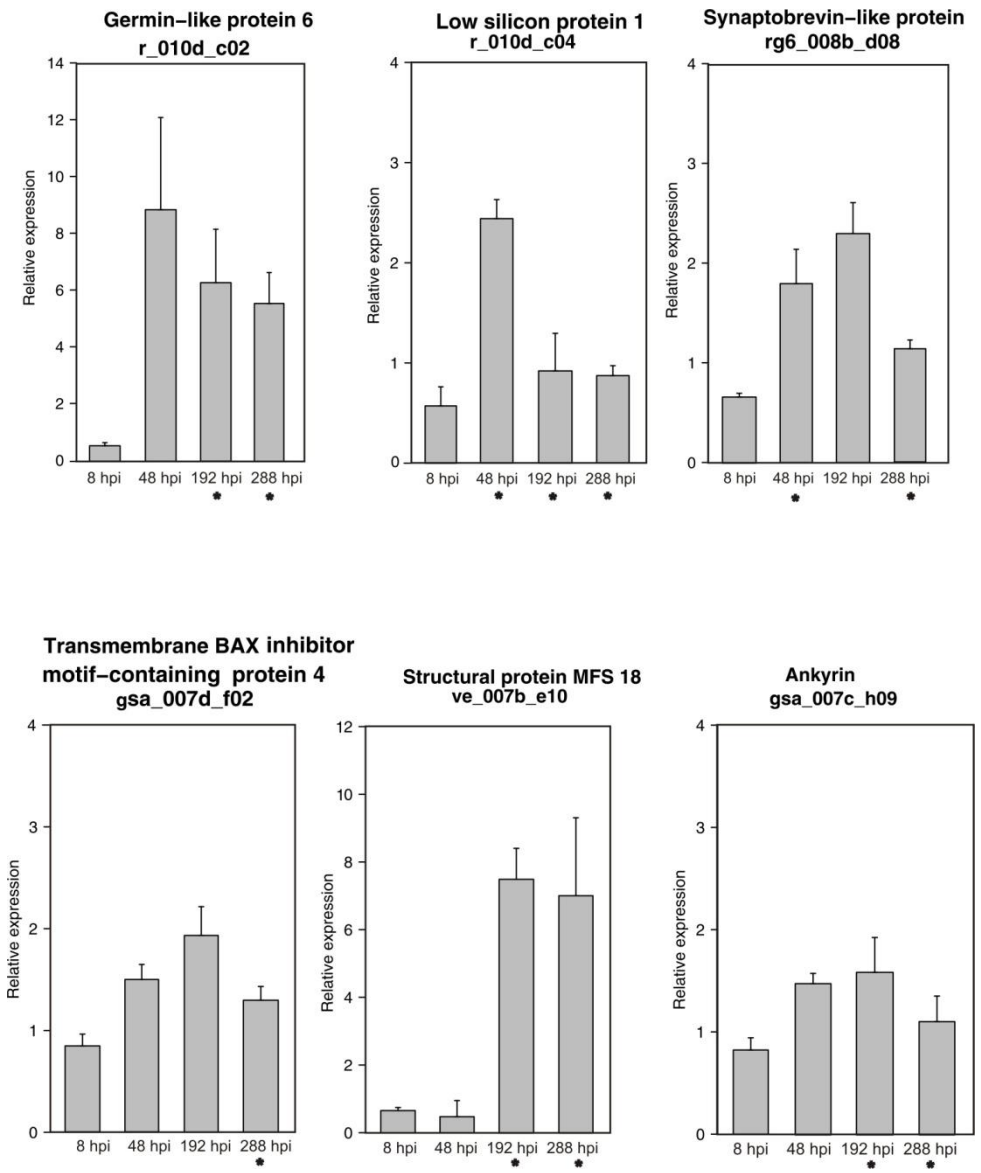

Figure 3.6 Quantitative real-time PCR analysis of genes differentially expressed in the partially resistant genotype after *Xtg* inoculation compared to the control treatment. Protein names above represent the annotation derived from GenBank and the blastx database (http://ncbi.nlm.nih.gov/BLAST/) and the sequence ID represents the unique identifier with information about the cDNA library (Asp et al. 2007) that the sequence was taken from. Error bars indicated standard errors and asterisks (*) indicate that the gene was significantly up-regulated according to the microarray analysis.

3.5 Discussion

Transcriptome analyses using a cDNA microarray developed from 9,990 unique EST sequences from perennial ryegrass (*Lolium perenne*) revealed 158 genes differentially

expressed in a resistant Italian ryegrass (*L. multiflorum*) genotype after inoculation with *Xanthomonas translucens* pv. *graminis* (*Xtg*) and 1,203 genes differentially expressed between a resistant and a susceptible *L. multiflorum* genotype. Cross-species hybridizations (CSH) in which the target cDNA and the cDNA spotted on the microarray are from different species have been shown to produce reliable results providing the phylogenetic distance between the two species in not too extreme (Gilad *et al.*, 2006). In this study, both the target species, and the species that was used for the development of the cDNA microarray, belong to the genus *Lolium* and are very closely related, as fully fertile F1 hybrids may be formed between the two species (Catalan *et al.*, 2004). Consistent signals were detected for an average of 45% of the spotted EST sequences. This is higher than the 25% to 35% reported for hybridization of *Arabidopsis halleri* cDNA to a microarray designed for *A. thaliana* (Becher *et al.*, 2004; Weber *et al.*, 2004) but comparable to the 45% to 52% that were achieved when hybridizing pepper (*Capsicum annuum*) and eggplant (*Solanum melongena*) cDNA to a microarray designed for tomato (*S. lycopersicum*; Moore *et al.*, 2005). In view of the close phylogenetic relationship of *L. multiflorum* and *L. perenne*, the observed level of cross-species hybridisation of 45% is rather low. However, the EST-sequences used for chip development were derived from cDNA libraries from a broad range of different tissues including roots, seed and leaves and a similar level of hybridization (50%) was observed in preliminary experiments using RNA from *L. perenne* leaves (data not shown).

The finding that no differentially expressed genes were detected 8 hours post inoculation (hpi) when comparing inoculation with *Xtg* and the control treatment is in agreement with the observations of Rechsteiner *et al.*, (2006), in which only three differentially expressed transcript derived fragments (TDF) were observed 12 hpi and no differentially expressed TDFs were detected before this time point using cDNA-AFLP. The rate of bacterial invasion may depend on the host–pathogen system (Wang & Sletten, 1995), recognition of bacterial effector proteins and the induction of the hypersensitive response (HR) usually occur within 24 hpi (Scheideler *et al.*, 2002). Thus, transcriptional changes leading to a HR were either absent or below the detection threshold of microarray and cDNA-AFLP analysis. It could also be that the host transcriptional reponse was manipulated by bacterial effectors initially suppressing host defences as it has been shown to occur during other *Xanthomonas* x host plant interactions (Kay & Bonas, 2009) or that there was no recognition of effector proteins secreted by *Xtg*.

Reproducibility of the results obtained with the cDNA microarray was demonstrated by verification of transcriptional changes using quantitative real-time PCR (qPCR; Fig. 3.6). The

cDNA microarray and the qPCR results were in good agreement with respect to trends of regulation. However, for some of the genes, identical patterns could not be reproduced as for example for rg6_008d_b08, which was significantly up-regulated at 48 hpi and at 288 hpi according to the microarray analyses, but showed the highest FC at 192 hpi according to qPCR analysis (Fig. 3.6, Supplementary Table S1). This could be due to the fact that qPCR is a very sensitive method for differential gene expression discovery of even very small amounts of transcripts (reviewed in Valasek & Repa, 2005) or due to a relatively stringent *p* value threshold ($p<0.01$) chosen in this study for the microarray experiments which failed to identify genuinely differentially expressed (false negatives) genes.

The comparisons between the resistant and the susceptible *L. multiflorum* genotype were based on non-isogenic, highly diverse genotypes. Differences in transcriptome profiles may therefore reflect general genetic differences and are not limited to differences related to resistance characteristics. Consequently, transcriptome analyses revealed 1,203 genes differentially expressed between the two genotypes, representing a wide range of different functions (Table 2.2). Although the use of genetically largely identical individuals derived from near isogenic lines would allow to reduce general expression differences, such individuals are difficult to obtain and often suffer from severe inbreeding depression (Posselt, 2010). The comparison of responses between a larger number of independently sampled genotypes from different phenotypic classes could present a valuable means to mitigate effects caused by disparate genetic backgrounds but may be restricted by limited resources available. The approach based on only two genotypes used in this study may serve as a proof of concept for further investigations. Interestingly, a remarkably high number of genes were differentially expressed between the resistant and the susceptible genotype after *Xtg* infection when compared to the number of genes differentially expressed after control treatment (Fig. 3.5). Further, in the susceptible genotype (29.5%) were up-regulated both after control treatment and *Xtg* inoculation, and 62.5% of the genes were detected to be up-regulated only after *Xtg* inoculation (Fig. 3.3). A much lower number (9.8%) was observed in the resistant genotype both after control treatment and *Xtg* inoculation and a higher percentage (84.1%) was observed only after *Xtg* inoculation. This indicates that gene expression in the resistant genotype is more variable in response to *Xtg* inoculation compared to the susceptible genotype. Further, the greatest gene expression differences between the two genotypes were observed at 48 hpi and the number of up-regulated genes in the susceptible genotype decreased between 48 hpi and 192 hpi (Fig. 3.5). The number of genes up-regulated in the resistant genotype after *Xtg* inoculation remained high (318) and did not decrease until 288

hpi, indicating that important genotype-specific processes potentially involved in defence responses occur between 48 hpi and 192 hpi. Additionally, more defence-related genes were up-regulated in the resistant genotype when compared to the susceptible genotype (Table 2). Up-regulated genes in the resistant genotype after *Xtg* inoculation may contribute to the phenotypic divergence in *Xtg* resistance between the resistant and the susceptible genotype. Genetic mapping of these up-regulated genes in the resistant genotype after *Xtg* inoculation compared to the susceptible genotype and subsequent expression QTL (eQTL) analyses is capable of assisting further elucidation of the genetic control of this variation as it has been shown for barley leaf rust in different barley genotypes or for stress response in different rice cultivars (Chen *et al.*, 2010; Inoue *et al.*, 2004).

Transcriptome analyses of the resistant genotype after *Xtg* inoculation revealed a number of differentially expressed genes previously reported to be involved in resistance and stress tolerance pathways. These genes represent candidate genes of particular interest for the development of tools for marker assisted resistance breeding. For example, the gene with the greatest fold change according to the microarray analyses (r_010d_c04) encodes Lsi1 belongs to a Nodulin26-like major intrinsic protein sub-family of aquaporins (Supplementary Table 1). Transcription of a Nodulin26-like major intrinsic protein has also been found to be up-regulated in citrus after infection with *X. axonopodis* pv. *axonopodis* (Cernadas *et al.*, 2008). Further, Lsi1 is known to be involved in silicon uptake in rice and barley, which is thought to be important for resistance against biotic and abiotic stress (reviewed in Ma & Yamaji, 2006). Silicon treatment has also been shown to reduce the chlorotic area of wheat leaves infected with the bacterial leaf streak causing pathogen *X. translucens* pv. *undulosa* (Silva *et al.*, 2010).

Another gene showed high sequence similarity to members of the family of germin-like proteins (GLP; r_010d_c02) which are known to be involved in broad-spectrum basal defence against various pathogens and are also induced upon abiotic stress (Manosalva *et al.*, 2009). Some germin-like proteins exhibit oxalate oxidase activity (Bernier & Berna, 2001). r_010d_c02 also revealed high sequence similarity to an oxalate oxidase mRNA from wheat, indicating that this GLP up-regulated by *Xtg* inoculation may also exhibit oxalate oxidase activity. In rice, a putative QTL for bacterial blight resistance caused by *X. oryzae* pv. *oryzae* (*Xoo*) mapped to chromosome 3 and was closely associated with the candidate gene oxalate oxidase (Ramalingam *et al.*, 2003). Chromosome 3 of rice displays conserved synteny with LG 4 of *L. multiflorum* (Devos, 2005) where a major QTL for bacterial wilt resistance was identified (Studer *et al.*, 2006). Further, an oxalate oxidase gene in *L. perenne* (*Lp*OXO) was

mapped to a similar location as the major QTL for bacterial wilt resistance on LG 4 of *L. multiflorum* (Dracatos *et al.*, 2009; Studer *et al.*, 2006).

Synaptobrevin-like transcripts such as rg6_008d_b08 which was up-regulated in the resistant genotype after *Xtg* inoculation have also been shown to be up-regulated in tomato and citrus infected with *Xanthomonas* spp. (Balaji *et al.*, 2007; Cernadas *et al.*, 2008). Synaptobrevin-like proteins are vesicle-associated membrane proteins involved in vesicle trafficking. The non-specific resistance genes *HvMLO* and *HvROR2* regulate accumulation of large vesicle-like structures in barley during powdery mildew attack (Collins *et al.*, 2003). Large vesicle-like particles contain small cell wall appositions, in which small vesicles accumulate between the plasma membrane and the cell wall (An *et al.*, 2006). Thus, polar vesicle trafficking has been shown to be involved in the formation of new cell-wall appositions which is important for basal defence (An *et al.*, 2006) presumably also supporting the mediation of partial resistance and defence to *Xtg*. Interestingly, rg6_008d_b08 also mapped to LG 4 in the VrnA mapping population (Studer *et al.*, 2010) indicating that this gene may be associated with the major QTL for bacterial wilt resistance observed on LG 4 (Studer *et al.*, 2006).

Expression of the flowering gene MFS18 (ve_007b_e10) was strongly induced in *L. multiflorum* leaves of the resistant genotype at 192 and 288 hpi with *Xtg*. MFS18 is a structural protein with sequence similarity to a gene expressed in male flowers (MFS18) of maize (Wright *et al.*, 1993). It has been previously documented that flowering time is strongly correlated with disease resistance, such that the expression of flowering genes is induced with progressing disease development (Collins *et al.*, 1999).

A gene encoding an ankyrin (ANK) repeat protein (gsa_007c_h09) was up-regulated after *Xtg* inoculation in *L. multiflorum* in the resistant genotype. The major role of plant ANK repeat proteins has mainly been related to signalling in defence and development mechanisms in *Arabidopsis* (Cao *et al.*, 1997). In pepper, the ANK domain C_3H_1 zink finger was not only up-regulated in response to infection with *X. axonopodis* pv. *glycines* but also as response to abiotic stresses such as cold and salt stress (Seong *et al.*, 2007). Therefore, the up-regulation of this gene encoding an ANK repeat protein may be associated with the specific signalling pathway triggered by *Xtg* infection.

In conclusion, the cDNA microarray developed using EST-sequences from *L. perenne* provides an efficient means to identify differentially expressed genes in *L. multiflorum* genotypes during pathogen infection. *L. multiflorum* defence responses detected by transcriptome analysis display many similarities to those of other species such as rice, cassava

and citrus after inoculation with *Xanthomonas* spp. Interestingly, the genes differentially expressed in the resistant *L. multiflorum* genotype after *Xtg* inoculation are remarkably similar to transcriptional changes triggered by pathogen associated molecular patterns (PAMPs) in other plant-pathogen interactions. Mapping of these candidate genes on the genetic linkage map of *L. multiflorum* developed by Studer et al., (2006) and subsequent QTL analyses may allow for the verification of genes co-locating with the major QTL on LG 4. Although the exact mechanism of putative *Xtg* resistance mediation by increased silicon transport needs to be further elucidated, the gene encoding for Lsi1 represents a promising candidate gene for marker assisted selection. The differentially expressed genes identified in this study represent a crucial element in understanding *Xtg* resistance in *L. multiflorum* and in the future may significantly facilitate the development of molecular markers as tools for resistance breeding. In addition, it was demonstrated that two genotypes with a contrasting level of *Xtg* resistance reveal substantial transcriptional difference especially at the 48 hpi and 192 hpi time points. The genes expressed at higher levels in the resistant genotype may be particularly useful to perform eQTL analyses in order to further understand the networks and pathways involved in *Xtg* resistance to identify genotypes with high levels of *Xtg* resistance.

3.6 Acknowledgements

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4 The *hrpG* gene of *Xanthomonas translucens* pv. *graminis* contributes to symptom development during bacterial wilt infection but is not essential for *in planta* survival

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4.1 Abstract

Xanthomonas translucens pv. *graminis* (*Xtg*) is the pathogen that causes bacterial wilt on a wide range of forage grasses including Italian ryegrass. The disease leads to considerable yield losses in forage production and breeding for resistance is currently the only applicable means to control the disease. The type III secretion system (T3SS) encoding a protein transport system for effectors is necessary for host colonization and pathogenicity in other phytopathogenic bacteria. A number of the genes encoding the T3SS are highly conserved among plant and animal pathogens. The two key regulators of the T3SS are the proteins HrpX and HrpG, representing a two-component regulatory system. Based on published sequences of *Xanthomonas* spp., we identified and sequenced the *hrpG* gene of *Xtg.* Using allelic exchange by homologous recombination, a *ΔhrpG* mutant of *Xtg* was obtained. Italian ryegrass plants inoculated with the *ΔhrpG* mutants showed significantly weaker symptoms compared to plants inoculated with the wildtype strain, indicating that the *hrpG* gene is important for disease development. Serial dilutions of infected plant material revealed that the *ΔhrpG* mutants are still able to survive and multiply inside the host. This is the first study of *Xtg* dealing with the T3SS and its expression activation with the *hrpG* gene.

4.2 Introduction

The pathogen *Xanthomonas translucens* pv. *graminis* (Egli et al., 1975) causes bacterial wilt of forage grasses, a disease leading to considerable yield losses depending on susceptibility of the host. The host range of *Xanthomonas translucens* pv. *graminis* (*Xtg*) includes various forage grass species (Egli & Schmidt, 1982). The pathogen invades the plant through wounded tissue and colonizes the xylem vessels resulting in symptoms such as wilting the leaves and necrosis of entire plants. Breeding for resistant cultivars based on recurrent phenotypic selection has lead to cultivars with improved resistance to bacterial wilt. Nevertheless, complete resistance has not been achieved and highly susceptible plants still occur after numerous cycles of recurrent selection (Michel, 2001). Therefore, understanding the processes involved in host colonization and bacterial wilt symptom development is a major objective and may provide necessary information for targeted resistance gene (*R*-gene) identification.

Xanthomonas spp. such as *Xoo* and other major Gram-negative phytopathogenic bacteria (e.g. *Pseudomonas syringae*, *Erwinia* spp., *Ralstonia solanacearum*) depend on the type III secretion system (T3SS) for host colonization (Galan & Collmer, 1999). The genes encoding the T3SS apparatus of phytopathogenic bacteria are organized in large gene clusters

on either the chromosome or a plasmid (Arnold *et al.*, 2003) and are termed *hrp* (hypersensitive response and pathogenicity) genes. Expression of the *hrp* gene cluster results in the formation of a membrane-spanning secretion apparatus called the Hrp-pilus, which mediates the delivery of bacterial effectors into the host cell (Cornelis & Van Gijsegem, 2000). These effectors can modulate the plant's physiology by interfering with resistance mechanisms of the plant or by facilitating nutritional and virulence processes of the pathogen (Büttner & He, 2009). At the same time, they represent essential determinants of pathogenicity on susceptible plants and are required for the induction of the hypersensitive response (HR) on resistant plants (White *et al.*, 2000). In various resistant plants, the specific recognition of effectors delivered by *Xanthomonas* spp. is mediated by *R*-genes and the elicitation of the HR is triggered in response to the recognition of these effectors. Transcription activation of the genes encoding the T3SS machinery and some effectors is driven by regulatory cascades that respond to the environment. Therefore, expression of genes encoding the *hrp* gene cluster has been reported to be suppressed in rich media but induced *in planta* or also may be induced in minimal media (Wei *et al.*, 1992). The first key component in the regulatory cascade responsible for expression of the *hrp* gene cluster is HrpG belonging, together with HrpX, to a two-component regulatory system (Noel *et al.*, 2001; Wengelnik *et al.*, 1996). HrpG is a member of the response regulator family of outer-membrane proteins (OmpR), whereas HrpX is an AraC-type transcriptional activator. Point mutations resulting in amino acid substitutions of the *hrpG* gene of *Xanthomonas campestris* pv. *campestris* and *X. c.* pv. *vesicatoria* resulted in the constitutive expression of the *hrp* gene cluster (Jiang *et al.*, 2006a; Wengelnik *et al.*, 1999) and has been used to identify effectors crucial for pathogenicity and symptom development. Mutants deficient of the *hrpG* gene of *Xanthomonas* spp. have not been able to induce a HR on resistant plants or create symptoms on susceptible plants anymore, resulting in the complete loss of pathogenicity (Cho *et al.*, 2008; Darsonval *et al.*, 2008; Wengelnik *et al.*, 1996; Zou *et al.*, 2006).

Since the T3SS is a major virulence factor of *Xanthomonas* spp., both the existence and the function of a hypothetical T3SS in *Xtg* has is of major importance for understanding in more detail the *L. multiflorum-Xtg* interaction. Therefore, the aims of the present study were to investigate the presence of genes encoding T3SS components by means of primer design based on the sequences of published *Xanthomonas* spp. and to investigate their role for type III secretion and pathogenicity of *Xtg.* In addition, we aimed at understanding *in planta* survival and multiplication of a T3SS mutant and to elucidate the importance of the *hrpG* gene for bacterial virulence by means of a mutant deficient of the *hrpG* gene.

4.3 Material and Methods

4.3.1 Overview

The *hrpG* gene of *Xtg*29 was sequenced using conserved primers designed on the consensus sequence of publicly available sequences of the *hrpG* gene of other *Xanthomonas* spp. Subsequent primer walking on genomic DNA resulted in a contig of 2,045 bp length. Two DNA fragments of approximately 500 bp of each of the two *hrpG* flanking regions were connected using Soeing PCR (Horton, 1995). The resulting fragment referred to as *ΔhrpG* fragment was cloned into the suicide vector pKNG101 resulting in the plasmid pCC101 (Fig. 4.1). The pKNG101 vector consists of the origin of replication of the plasmid R6K (oriR6K) and due to oriR6K, the suicide vector pKNG101 (Kaniga *et al.*, 1991) is unable to replicate extrachromosomally in the bacterial host unless it produces the λ protein encoded by the *λpir* gene. Therefore, the *Escherichia coli* K12 SM10(λpir) strain was used for suicide plasmid replication. In addition, the vector contains the streptomycin resistance (*strAB*) gene, a gene encoding the origin of transfer (mobRK2), the *sacB* gene mediating sucrose sensitivity and a multiple cloning site (Fig. 4.1).

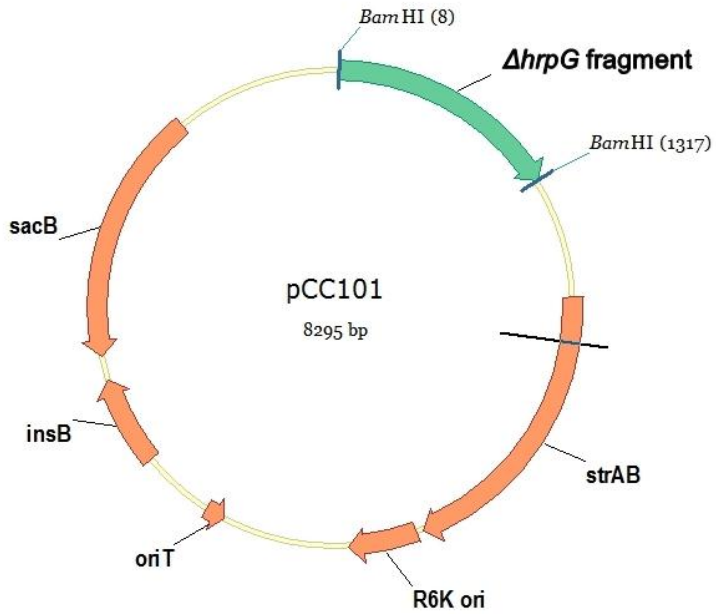


Figure 4.1 The suicide vector pCC101 derived from the pKNG 101 vector containing the *ΔhrpG* fragment

Electro-competent *Xtg*29 were transformed with the suicide vector pCC101 (Fig. 4.1) containing the *ΔhrpG* fragment. Successfully transformed *Xtg*29 containing the integrated suicide vector in the genome were selected with streptomycin. Double homologous

recombination leading to a *hrpG* deletion was induced after culturing in media devoid of streptomycin and plating on sucrose plates. Due to the *sacB* gene on the suicide vector, *Xtg* still containing the integrated copy of pKNG101 were sucrose sensitive and did not grow on plates containing sucrose. The resulting *ΔhrpG* mutant was verified by PCR. In order to screen for virulence and test *in planta* survival and multiplication, *Lolium multiflorum* plants were infected with the *ΔhrpG* mutant and the wildtype strain. Scoring for bacterial wilt symptoms and re-isolation from plant material were used to compare disease symptoms and *in planta* survival.

4.3.2 Bacterial isolates and cultivation conditions

The bacterial isolates and plasmids used in this study are listed in Table 4.1. *X. translucens* pv. *graminis* isolate 29 (*Xtg*29) has previously been characterized for virulence on different genotypes and cultivars (Kölliker *et al.*, 2006; chapter 2). Until usage, *Xtg29* was stored at -70ºC in GY broth (glucose 2%, yeast extract 1%) containing 15% glycerol. *Xtg* was grown in Circle Grow (CG) broth (Molecular Probes, Eugene, OR, USA), on CG plates containing 1.5% bacto agar or on GYC plates (glucose, yeast extract, $CaCO_3$ 2% and 1.5% agar) at 28ºC. *E. coli* cells were cultivated in Luria-Bertani broth (LB; 1% bacto tryptone, 0.5% bacto-yeast extract, 0.5% sodium chloride) or on LB plates containing bacto-agar (1.5%) at 37ºC. Antibiotics were used at following concentrations: 50 µg/ml ampicillin, 50 µg/ml kanamycin, 25 µg/ml streptomycin and 300 µg/ml rifampicin.

Table 4.1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characterisitcs	Source or reference
pKNG101	sucide vector; Strep^r	Kaniga *et al.*, (1991)
pDSK-GFPuv	plasmid vector; Kan^r	Wang *et al.*, (2007)
pCC101	pKNG101 derivative containing the *ΔhrpG* fragment	this study
Xtg 29	*X. translucens* pv. *graminis*	Kölliker *et al.*, (2006)
Xtg 29 (Rif^r)	*X. translucens* pv. *graminis*; Rif^r	this study
Xtg 29Δ*hrpG*	*X. translucens* pv. *graminis*; *ΔhrpG*; Rif^r	this study
Xtg 29Δ*hrpG*+*hrpG* (a)	*X. translucens* pv. *graminis* transformed with pFW007; Kan^r	this study
Xtg 29Δ*hrpG*+*hrpG* (b)	*X. translucens* pv. *graminis* transformed with pFW008; Kan^r	this study
K12 SM10	*Escherichia coli* wildtype strain, λ pir, Kan^r	Miller *et al.*, (1988)
pFW007	Used for complementation of the *hrpG* gene; pDSK-GFPuv derivative containing the *hrpG* coding sequence including own promoter; Kan^r	this study
pFW008	Used for complementation of the *hrpG* gene; pDSK-GFPuv derivative containing the *hrpG* coding sequence under PpsbA promoter; Kan^r	this study

To prepare electro-competent *Xtg*, the cells were cultured in 100 ml CG medium up to an OD_{600} of 0.6-0.8 in a rotary shaker at 28ºC and 150 rpm. The cells were harvested by centrifugation for 10 min at 4500 rpm and 4ºC and washed twice with 100 ml 15% cold glycerol. After centrifugation at 4500 rpm for 10 min 4ºC, the supernatant was removed and the cells were resuspended in 300 µl of 15% glycerol. 50 µl of competent cells were used for transformation using a MicroPulser (Bio-Rad, Reinach, Switzerland) and the EC2 program in 0.2 cm cuvettes (Bio-Rad). 350 µl of SOC medium was added to the transformed *Xtg* cells and incubated at 28ºC for 2 hours in a rotary shaker. 100 µl of cells were plated on CG or GYC plates supplemented with antibiotics when required. Sucrose (5%) was added to the media when selecting for the second crossing-over event.

4.3.3 Primer design, plasmid isolations and PCR conditions

Plasmid isolations were performed with the PureYield™ Plasmid Miniprep System (Promega, Madison, WI, USA). Restriction enzymes were used according to the manufacturer's recommendations (New England Biolabs, Ipswich, MA, USA). Cloning reactions were performed using the Dephos and Ligation kit (Roche, Penzberg, Germany). *HrpG* primers (Table 2) were designed based on conserved sequences of the following published xanthomonads: accession NC_010717 of *X. oryzae* pv. *oryzae* isolate PXO99A (Salzberg *et al.*, 2008), accession NC_010688 of *X. campestris* pv. *campestris* B100 (Vorhölter *et al.*, 2008), accession NC_007508 of *X. c.* pv. *vesicatoria* str. 85-10 (Thieme *et al.*, 2005), accession NC_003919 of *X. axonopodis* pv. *citri* str. 306 (Da Silva *et al.*, 2002). PCR reactions were performed using the primers listed in Table 4.2. PCR reactions were conducted in 20 µl volumes using Hotstar DNA Polymerase (Qiagen, Hilden, Germany) or Phusion (Finnzymes, Espoo, Finland) depending on the need for a polymerase with proofreading activity. The PCR conditions for Hotstar DNA Polymerase were as follows: initial denaturation at 94ºC for 15 minutes; followed by 35 cycles of 94ºC for 30 seconds, annealing was performed according to primer design between 50ºC and 60ºC for 40 seconds, extension was performed 1 minute per 1 kb. Following the 35 cycles of amplifications, a final extension step of 7 minutes at 72ºC was conducted. PCR conditions for Phusion (Finnzymes) were: initial denaturation at 98ºC for 1 minute; followed by 35 cycles of 94ºC denaturation for 10 seconds, annealing was performed according to primer design between 50ºC and 60ºC for 20 seconds, extension was performed 1 minute per 1 kb. Following the 35 cycles of amplification, a final extension step of 7 min at 72ºC was conducted. Sequencing was performed using gene specific primers or M13 forward or M13 reverse, BigDye™ v. 1.1 (Applied Biosystems, Foster City, CA, USA) and an ABI3130xl (Table 4.2).

Table 4.2 Primer sequences and their application used in this study. Underlined sequences indicate restriction enzyme sites used for cloning.

Primer	Target gene, vector or application	Sequence (5'→3')
hrpG_F400	hrpG sequencing	GCMTGGCAGCGCTGCCAYAG (Müller-Hug, 2008)
hrpG_R791	hrpG sequencing	TCCATGGTGCGGTCGGTGAA (Müller-Hug, 2008)
hrpG2	hrpG sequencing	CGAGCAGATAGGCTCCCACAA
hrpG3	hrpG sequencing	GAACGATGAGCCGATCAAGCTC
hrpG9	hrpG sequencing	GACAATGGCGCGTCCTAGCA
hrpG17	hrpG sequencing	CCAACCTGTGCACCACGCTCT
hrpG5	hrpG verification	CGGAATGCTGAGCACATCCAG
hrpG13	hrpG verification	CAGGCCGTTGCGGTCACGCAC
hrpG12	hrpG verification	CGCATGGCTACCAGCTCGAGC
hrpG16	hrpG verification	ACGCTCTACGGCACATGGGC
PrComp1	hrpG Soeing PCR for complementation	CGGAATTCCATCCGCCGCCGACTGGCGG
PrComp2	hrpG Soeing PCR for complementation	AAACTGCAGGATGCGGAGGCGTCCAGCGC
PrComp3	hrpG Soeing PCR for complementation	CGGAATTCGAGCTCGGTACCCGGGGATC
PrComp4	hrpG Soeing PCR for complementation	ATGTATATCTCCTTCTTAAAGTTAAAC
PrComp5	hrpG Soeing PCR for complementation	TTTAAGAAGGAGATATACATGTCGACATGATGAGTCCGGATTGGGC
hrpG_SoePr1	Soeing Primer 1 *hrpG*	CGGGATCCACGCTCTACGGCACATGGGC
hrpG_SoePr2	Soeing Primer 2 *hrpG*	GGTGCCCAATCCGGACTCAT
hrpG_SoePr3	Soeing Primer 3 *hrpG*	ATGAGTCCGGATTGGGCACCCCTGGCCAGCGCCTAAGCCC
hrpG_SoePr4	Soeing Primer 4 *hrpG*	CGGGATCCCCGGCCAGCGGCAGCAGCGC
pKNG101_F1	pKNG101	GCCATCAAACCACGTCAAAT
pKNG101_R1	pKNG101	CCGGATGCTGGTAAAGCTAC
M13 forward	Sequencing of subcloned PCR fragments	TGTAAAACGACGGCCAGT
M13 reverse	Sequencing of subcloned PCR fragments	CAGGAAACAGCTATGACC
16SV1-Xan(f)	ribosomal RNA	AGCACAGTGGTAGCAATACCATG
EUB338-rev	ribosomal RNA	GCTGCCTCCCGTAGGAGT

The *ΔhrpG* fragment used for site-directed mutation of the *hrpG* gene was generated using Soeing PCR (Horton, 1995) by connecting the PCR fragments amplified from the *hrpG* flanking regions and with primers containing the sequence of a BamHI restriction site at the 5' end of each primer. PCR fragments were subcloned into the pCR®4Blunt-TOPO® vector (Invitrogen, Carlsbad, USA). The *ΔhrpG* fragment was then ligated into the suicide vector pKNG101 (Kaniga *et al.*, 1991). Control and selection of single and double crossing-over events were performed with PCR on single colonies and the primers listed in Table 4.2. Mutants deficient of the *hrpG* gene were complemented with the *hrpG* gene. Therefore, the

GFPuv gene of the plasmid pDSK-GFPuv (Wang *et al.*, 2007) was excised by digestion with *EcoRI* and *PstI* and the coding region of *hrpG* was inserted into the vector.

4.3.4 Screening for virulence of the *ΔhrpG* mutant

The highly susceptible *L. multiflorum* genotype *LmK*-01 (chapter 3) was used for virulence screening of the *Xtg29* rifampicin resistant strains of the wildtype and the *ΔhrpG* mutant, and the two different complemented *Xtg29 ΔhrpG* isolates (kanamycin resistant). A negative control treatment consisted of cutting the plants without inoculum. Assessment of bacterial wilt symptoms was performed using four replications per genotype x treatment combination in a completely randomized block design. Scoring for bacterial wilt symptoms was performed 7, 14, 21 and 28 days post infection (dpi) according to a scale ranging from completely healthy (1) to dead (9) with intervals as described in chapter 2. The plants were clonally propagated by separating single tillers, which were transferred into pots and were inoculated using the leaf clipping technique described by Kölliker *et al*., (2006). The inoculated plants re-grew in the greenhouse at an average of 20ºC/ 18ºC (day/night temperatures), an average of 70% relative humidity and long day conditions (16 h light, [>100µE/m^2s]).

4.3.5 Generation of rifampicin resistant *Xtg*29 and *Xtg*29 *ΔhrpG*

In order to be able to selectively re-isolate *Xtg29* and *Xtg29 ΔhrpG* mutants from plant material, rifampicin resistance was induced in these two strains. Rifampicin was dissolved in methanol to a concentration of 10 mg/ml. In the bacterial cultures, the concentration of rifampicin was continuously increased in CG liquid media from initially 10 µg/ml over intervals of 30 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml to a final concentration of 300 µg/ml in a rotary shaker at 28˚C and 150 rpm. One ml of bacterial culture was used to inoculate 4 ml of CG media supplemented with the required amount of rifampicin. Cells were grown for approximately 24 hours until the rifampicin concentration was increased.

4.3.6 *In planta* multiplication of *Xtg* and *Xtg*29 *ΔhrpG*

For determination of *in planta* multiplication and bacterial population densities, plants of the genotype *Lm*K-01 were grown in the growth chamber at 19ºC/23 ºC (average day/night temperature), a relative humidity of 80-85% and long day conditions (16 h light [350 µmol/m^2s) after inoculation or control-treatment. In order to ensure constant temperature and light conditions, plants were grown in the growth chamber. Scoring of bacterial wilt symptoms was not performed in the growth chamber, as the symptoms that occur in the growth chamber are different from symptoms observed in the greenhouse and no applicable

scoring scale has been developed. For determination of colony forming units (CFU) per g of fresh plant material during the early disease stages, leaves and tillers were harvested at four different time points after infection: 6 hours post infection (hpi), 4, 7 and 14 days post infection (dpi). A second experiment was performed in order to determine the CFU/g plant material at later stages of bacterial wilt infection (i.e. 14, 17, 21 and 28 dpi). For serial dilution plating, entire plants were cut 2 cm above soil. Surface sterilization was performed by incubating the plant material in 1% Chloramine-T solution (Honeywell Riedel de-Haën, Seelze, Germany) for 10 min. After incubation for 2 min. in sterile water, the material was homogenized in NaCl-solution (0.9%). Serial dilutions were prepared on CG medium (Molecular Probes) plates supplemented with 300 µg/ml of rifampicin (AppliChem, Darmstadt, Germany). Bacterial cell counts per g of fresh plant material were determined after incubation of the plates at 28ºC for 7 days. In order to verify the identity of the isolated bacteria, PCR using primers targeting the *hrpG* gene (Table 4.2) was conducted on ten different colonies per treatment and time point.

4.3.7 Data analyses and statistics

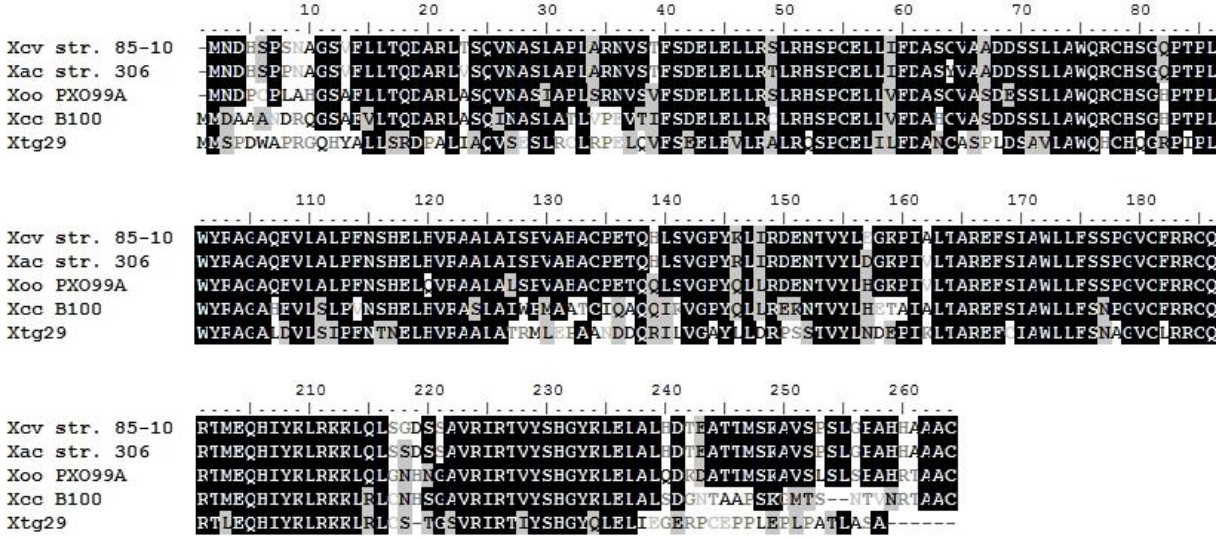
The area under the disease progress curve (AUDPC) values were calculated for each inoculated plant from the scores obtained at 7, 14, 21 and 28 days after infection (chapter 2) following the formula ($AUDPC = \sum_i [(Y_i + Y_{i-1}) \times (t_i - t_{i-1})] / 2$) where Y_i is the disease score at time point i using the scores 1 to 9 and t_i is the number of days after inoculation. In order to compare the AUDPC values caused by the individual isolates, a multiple t-test was performed. The CFU data for determination of *in planta* multiplication was compared with a separate two-sided t-test for each time point individually. *P*-values<0.05 were considered to be significant. All statistical analyses were performed in R (The R Development Core Team, 2008) using the packages: stats, graphics and coin (Hothorn *et al.*, 2006).

4.4 Results

4.4.1 Sequence analysis of HrpG

In order to characterize the role of *hrp* genes for *in planta* survival and multiplication of *Xtg*, primers on the consensus sequence of the *hrpG* gene of published *Xanthomonas* spp. were designed. Sequencing of resulting PCR products and subsequent primer walking resulted in an assembled contig of 2,045 bp length containing the entire coding sequence. Blastx search (http://blast.ncbi.nlm.nih.gov/) of the sequenced region and multiple sequence alignment using CLUSTALW revealed a sequence homologous to the *hrpG* gene of *Xanthomonas* spp. (Fig. 4.2A). The coding sequence consisted of 774 bp (65% GC content) length and 258 amino acids. The predicted HrpG protein sequence of *Xtg*29 revealed pairwise identities of 51.8% to *X. campestris* pv. *vesicatoria* str. 85-10 (*Xcc*) and *X. oryzae* pv. *oryzae* PXO99A (Xoo), 52.2% to *X. axonopodis* pv. *citri* str. 306 (*Xac*) and 52.6% to *X. c*. pv. *campestris* B100 (*Xcc*; Fig. 4.2B). The flanking regions of the *hrpG* gene revealed no significant similarity to the nucleotide sequences of the other *Xanthomonas* spp. mentioned above (data not shown).

A)



B)

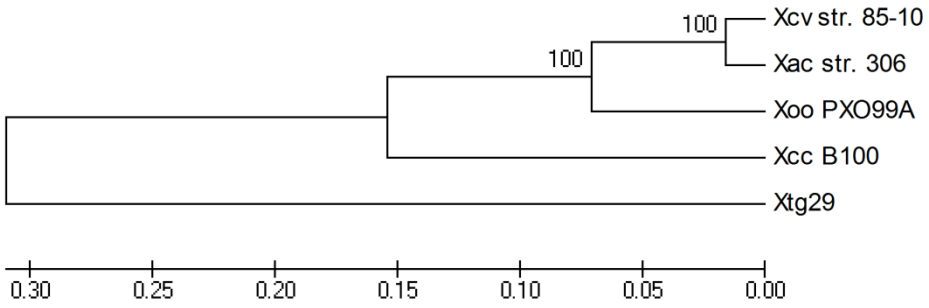


Figure 4.2 Comparison of HrpG proteins between sequenced *Xanthomonas* spp. and *Xanthomonas translucens* pv. *graminis* (*Xtg*29). A) Multiple sequence alignment (CLUSTALW) of the predicted HrpG protein sequence of *Xtg*29 compared to other sequences presented of *Xanthomonas* spp. The abbreviations with whole genome sequence accession numbers of GenBank in parenthesis are as follows: Xcv: *X. campestris.* pv. *vesicatoria* str. 85-10 (**NC_007508**), Xac: *X. axonopodis* pv. *citri* str. 306 (**NC_003919**), Xoo: *X. oryzae* pv. *oryzae* PXO99A (**NC_010717**), Xcc: *X. c.* pv. *campestris* B100 (**NC_010688**). Gaps in the alignment are indicated with dashes. B) Neighbor-joining bootstrap tree for amino acid sequences of the HrpG protein of *Xtg*29 compared to other *Xanthomonas* spp. Numbers above branches indicate bootstrap values >50% based on 1000 permutations.

4.4.2 Generation of a *ΔhrpG* mutant of *Xtg*

A strain of *Xtg*29 deficient of the *hrpG* gene was generated using double homologous recombination after transformation with the pCC101 plasmid (Fig. 4.1) and selection on media containing 5% sucrose. The different recombination events were verified using PCR and hrpG specific primers (Table 4.2). The first recombination event was verified using the primers hrpG12 and hrpG13 resulting in a fragment size of 151 bp (Fig. 4.3, lane 1), hrpG12 and pKNG101_R1 resulting in a fragment size of 705 bp (lane 2, Fig. 4.3), hrpG17 and

pKNG101_R1 resulting in a fragment size of 560 bp (Fig. 4.3, lane 3) and pKNG101_F1 and hrpG5 resulting in a fragment size of 1188 bp (Fig. 4.3, lane 4).

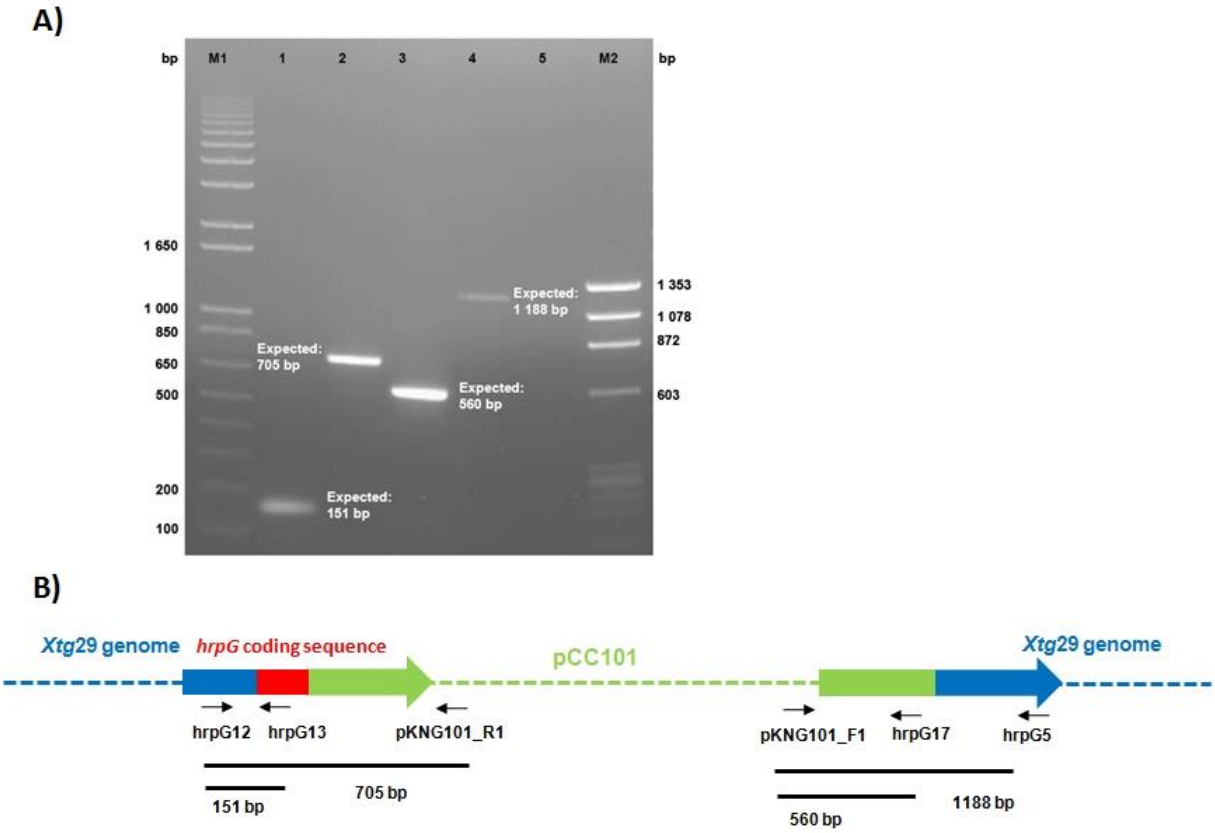


Figure 4.3 A) PCR verification of the first recombination event. M1: 1kb plus DNA Ladder, M2: Hae III fragments (Invitrogen). Lanes 1 – 5: PCR reactions performed with different primer pairs and their expected amplified fragment sizes. B) Map of first recombination event, primer annealing positions and expected fragment sizes.

The second homologous recombination event was induced by growing the *Xtg* cultures from the first recombination event devoid of streptomycin. Single colonies were screened and verified using the primers hrpG16 and hrpG13. PCR reactions on *ΔhrpG* mutants amplified a fragment of 776 bp length (Fig. 4.4, lanes 1 of *ΔhrpG* mutants), whereas PCR on the strains that recombined back to the wildtype (revertant) amplified a fragment of 1531 bp length (Fig. 4.4, lane 1 of *hrpG* revertant). The primer combination hrpG12 and hrpG13 that amplified a fragment of 151 bp in the wildtype strain (Fig. 4.4 lane 2 of *hrpG* revertant) and did not amplify anything in the *ΔhrpG* mutants (Fig. 4.4, lane 2 of *ΔhrpG* mutants). PCR using the primers hrpG17 and pKNG101_R1 verified the second homologous recombination event that excised the vector sequence from the genome not amplifying any product (Fig. 4.4, lanes 3).

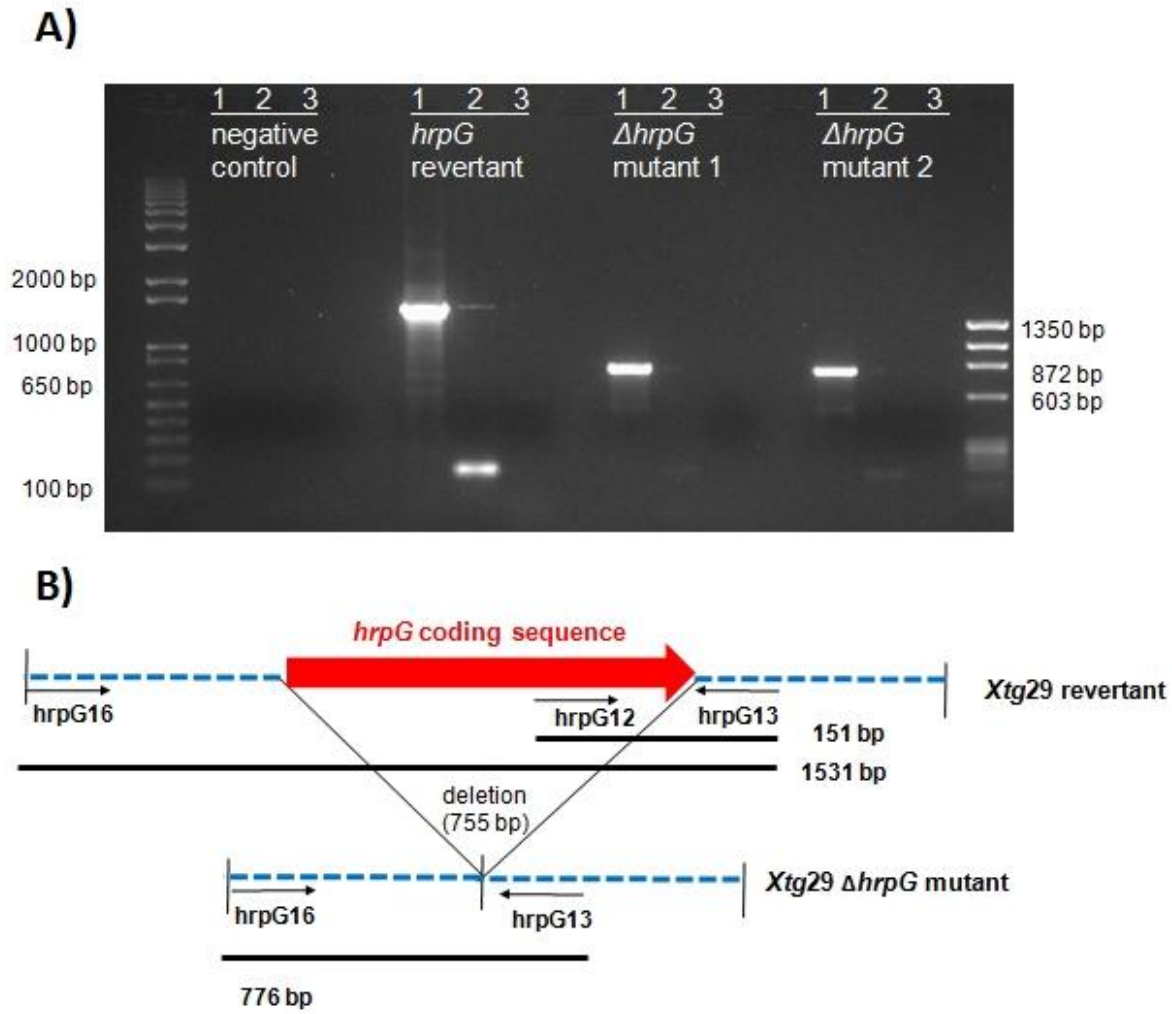


Figure 4.4 A) Gel electrophoresis of PCR products from colonies of the second homologous recombination event after cultivation on media containing sucrose. Primer combinations: 1: primers hrpG16 and hrpG13, 2: primer hrpG12 and hrpG13, 3: hrpG17 and pKNG101_R1. B) Map of second recombination event of the *Xtg*29 revertants and *ΔhrpG* mutants. Primer annealing positions are indicated with arrows.

4.4.3 Phenotype of *ΔhrpG* mutants

In vitro growth of the *ΔhrpG* mutants and both of the complemented *ΔhrpG* mutant strains was not affected by the *hrpG* mutation or the presence of an extrachromosmal plasmid on GYC plates and the colonies could not be distinguished from the wildtype strain (Fig. 4.5).

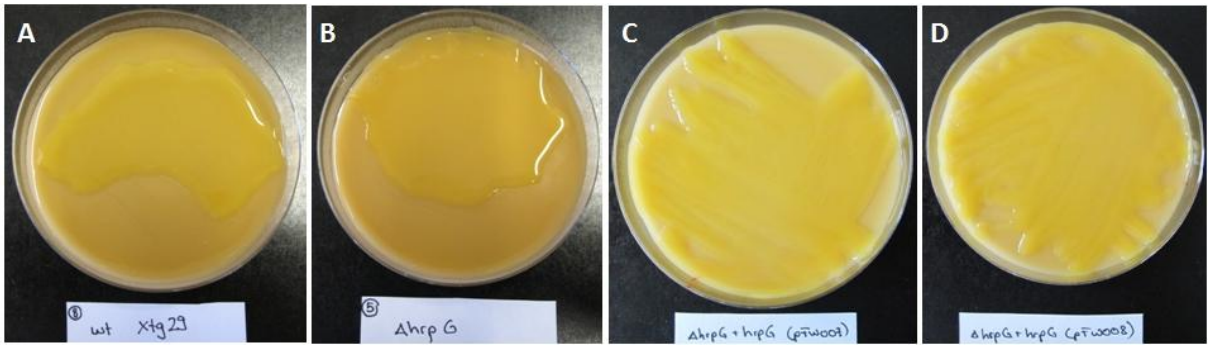
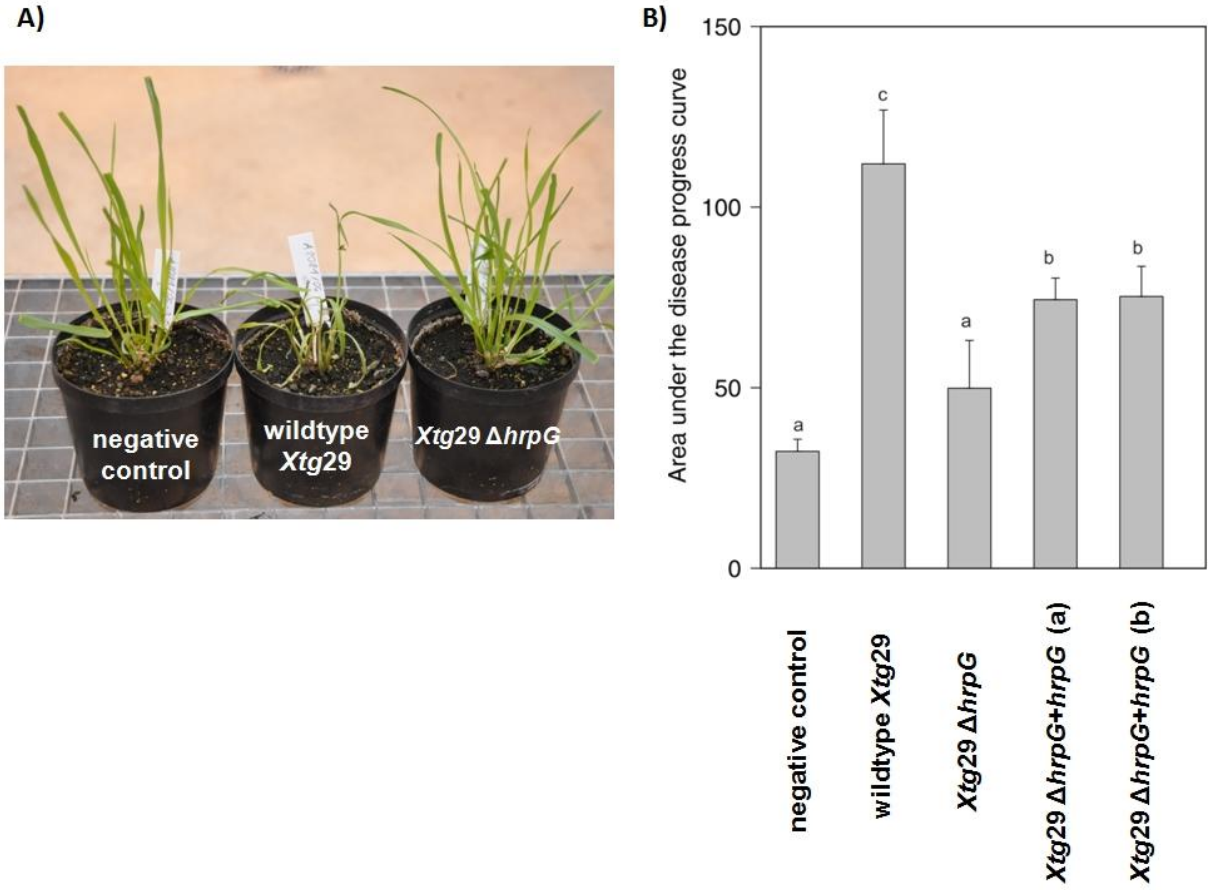


Figure 4.5 A) Colony morphology on glucose yeast calcium carbonate (GYC) agar plates of the *Xanthomonas translucens* pv. *graminis* (*Xtg*29) wildtype strain, B) Colony morphology on GYC agar plate of *ΔhrpG* mutant, C) Colony morphology on GYC agar plate containing kanamycin of the *ΔhrpG+hrpG* complemented strain under the native promoter (transformed with plasmid pFW007), D) Colony morphology on GYC agar plate containing kanamycin of the *ΔhrpG+hrpG* complemented strain under the constitutively expressed promoter PpsbA (transformed with plasmid pFW008).

After propagation on GYC plates, the *Xtg* mutants deficient of the *hrpG* gene were tested on the highly susceptible *L. multiflorum* genotype *Lm*K-01 (Fig. 4.5A and B) in the greenhouse. The symptoms caused by the *ΔhrpG* mutants were significantly weaker than the symptoms caused by the wildtype *Xtg*29 isolate (Fig. 4.6 A). Average disease scores ranging from 1 to 9 (chapter 2) for the plants infected with the wildtype isolate of *Xanthomonas translucens* pv. *graminis Xtg*29 increased from 2.00±0.82 at 7 days post inoculation (dpi), to 5.00±0.00 at 14 dpi, to 6.00±1.15 at 21 dpi and 6.00±0.82 resulting in an average area under the disease progress curve (AUDPC) value of 112.00±14.85 (Fig. 4.6 B). On the other hand, disease scores of the plants infected with the *ΔhrpG* mutant ranged from 1.75±0.50 at 7 dpi, to 1.75±0.96 at 14 dpi, 2.25±0.96 at 21 dpi, and 2.75±0.50 at 28 dpi, respectively, resulting in an average AUDPC value of 49.88±5.98. Control-treated plants were described with disease scores 1.00±0.00 at 7 dpi, 1.00±0.00 at 14 dpi, 1.5±0.58, at 21 dpi and 2.25±0.50 at 28 dpi, respectively, resulting in an average AUDPC value of 32.28±3.35.

Disease scores of the *ΔhrpG* mutants complemented with the *hrpG* gene under the native *hrpG* promoter were 1.75±0.50 at 7 dpi, 3.75±0.50 at 14 dpi, 3.5±0.58 at 21 dpi and 3.25±0.50 at 28 dpi resulting in an average AUDPC value of 74.38±5.98. Scores of the *ΔhrpG* mutant complemented with the *hrpG* gene under the constitutively expressed promoter PpsbA were 2.00±0.50 at 7 dpi, 3.25±0.50 at 14 dpi, 3.5±0.58 at 21 dpi and 4.00±0.50 at 28 dpi resulting in an average AUDPC value of 75.25±8.33. The *ΔhrpG* mutant strain complemented with the *hrpG* gene under the native promoter and the constitutively expressed PpsbA

promoter caused disease symptoms with AUDPC values corresponding to 39.44% and 40.84% of the AUDPC values caused by the wildtype strain.



A): complemented *hrpG* gene with own promoter (pFW007 plasmid)

B): complemented *hrpG* gene with PpsbA promoter (pFW008 plasmid)

Figure 4.6 A) Phenotype of the *ΔhrpG* mutant. One plant was cut without inoculum (left), one plant was inoculated with the wildtype *Xtg*29 isolate (middle) and one plant was inoculated with the *Xtg*29 *ΔhrpG* mutant isolate (right). The plant inoculated with the wildtype isolate showed severe disease symptoms 7 days after infection. B) Area under the disease progress curve (AUDPC) values of a highly susceptible *Lolium multiflorum* genotype either after control-treatment or inoculated with the *Xanthomonas translucens* pv. *graminis* wildtype isolate *Xtg*29, the *ΔhrpG* mutant or the *ΔhrpG* mutants complemented with the *hrpG* gene under the native promoter (a) or the PpsbA promoter (b). The data were collected at 7, 14, 21 and 28 days after inoculation. Different letters indicate significantly different AUDPC values according to a pairwise t-test (P<0.05) determined on four replications. P-value adjustment was performed according to Holm (1979).

4.4.4 Bacterial multiplication

In order to characterize the role of the *hrpG* gene for survival and multiplication of *Xtg* in *L. multiflorum* leaves, the dynamics of bacterial population densities for the wildtype strain (*Xtg*29) and the *ΔhrpG* mutant was determined using the rifampicin resistant *Xtg29* isolate and the *Xtg29 ΔhrpG* mutants. Despite the same initial inoculum concentration (OD_{600}=0.6), population densities of the *ΔhrpG* mutant quantified in leaves at 6 hours post infection were about 0.9-fold those of the wildtype isolate *Xtg*29 (Fig. 4.7). At 4 days post infection (dpi), both isolates multiplied and reached average $\log_{10}$ population densities per g of fresh weight of 8.72 for the wildtype and 8.07 for the *ΔhrpG* mutant. After this time point, a further increase in population densities followed until 14 dpi. Significant differences were observed at 4 and 14 dpi (P<0.05) during the first experiment monitoring the CFU data during early time points after infection. In the second experiment, average $\log_{10}$ CFU counts per g of fresh weight remained stable at 9.90, 9.28, 0.09 and 9.38 for the wildtype strain and 9.07, 8.64, 8.53 and 8.07 for the *ΔhrpG* mutant throughout all time points (14, 17, 21 and 28 dpi) and significantly lower population densities of the *ΔhrpG* mutant were recorded only at 28 dpi although the population densities of the *ΔhrpG* mutant remained stable (P<0.05). Of each treatment, ten colonies were screened by colony PCR to contain the expected genotype using the hrpG5 and hrpG16 primers (Table 4.2) for the wildtype *Xtg*29 isolate, and the hrpG13 and hrpG16 primers for the *ΔhrpG* mutant. *Xtg* could not be isolated from any of the negative control plants (only cut without inoculum). No cross-contamination was observed and the expected mutation remained stable throughout the observed time range.

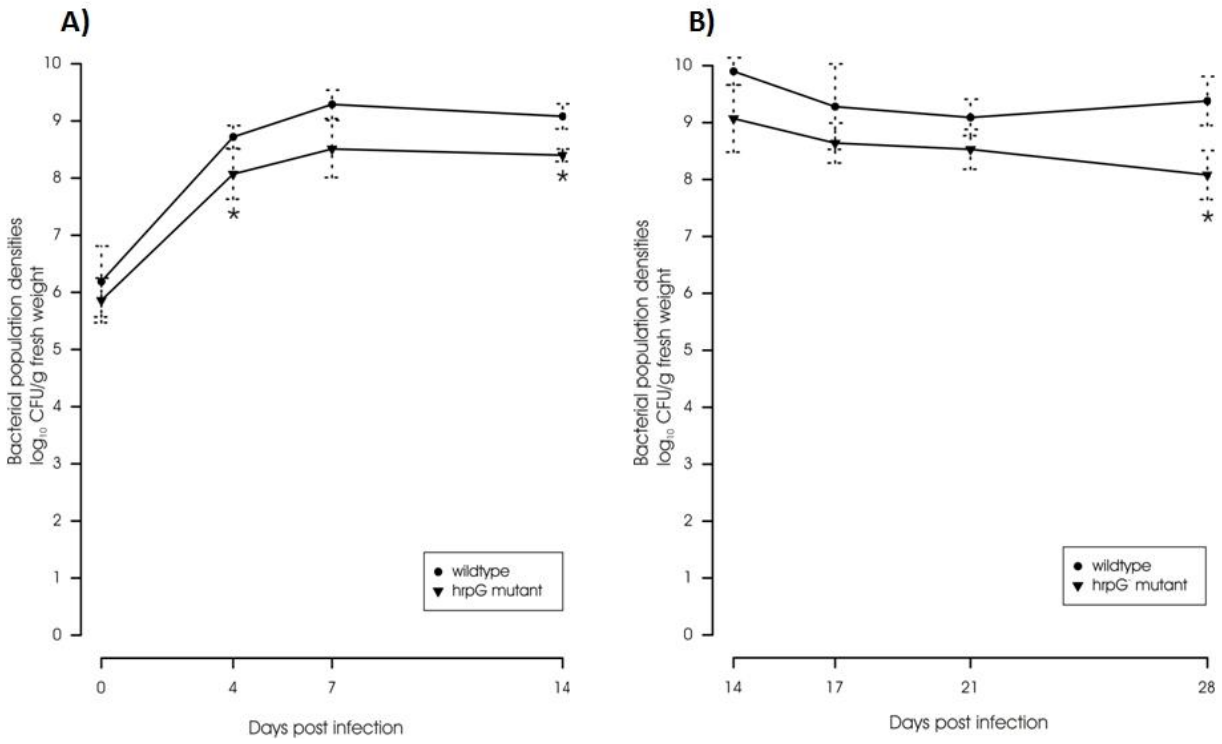


Figure 4.7 Colonization of *Lolium multiflorum* by *Xanthomonas translucens* pv. *graminis* 29 (*Xtg*29) and the *Xtg*29 deficient of the *hrpG* gene A) Bacterial population densities at early stages after infection were determined on leaves of three different plants sampled at 6 h and 4, 7, and 14 days post infection (dpi). B) Bacterial population densities at late stages after infection were determined on leaves of four different plants sampled at 14, 17, 21 and 28 dpi. Means and standard deviations were calculated from the three or four replicates per sampling date, respectively. Mean population densities with asterisks (*) are significantly (P<0.05) different on the basis of a two-sided t-test.

4.5 Discussion

A gene responsible for the transcription activation of the genes encoding the T3SS components has been shown to be present in *Xtg* and to be involved in the colonization process of *Xtg* in forage grasses. The fact that symptom development is impaired by site-directed mutagenesis of the *hrpG* gene in *Xtg* demonstrates that *Xtg* most likely depends on a T3SS for pathogenicity. Mutant studies with other *Xanthomonas* spp. deficient of the *hrpG* gene have shown that establishing high bacterial population densities in plant leaves was impaired, and for these species resulted in complete loss of pathogenicity (Cho *et al.*, 2008; Darsonval *et al.*, 2008). However, the present *ΔhrpG* mutant of *Xtg* still caused detectable disease symptoms when compared to the negative control (Fig. 4.6). However, this difference was not significant according to the t-test (P>0.05). This phenomenon was also observed for *X. oryzae* pv. *oryzicola* (Zou *et al.*, 2006) isolates deficient of the *hrpG* gene. The *ΔhrpG* mutant of *X. oryzae* pv. *oryzicola* was not able to induce the hypersensitive response (HR) anymore during the incompatible interaction or cause water soaking lesions during the compatible interaction in rice seedlings. At the same time, it retained weak pathogenicity when inoculated into leaves of adult rice plants by means of leaf infiltration.

On the other hand, *X. fuscans* pv. *fuscans* mutants deficient of the regulatory *hrp* genes (*hrpX* or *hrpG*) were more affected than mutants deficient of the genes encoding any of the structural components of the T3SS (Darsonval *et al.*, 2008). In this study, the $\log_{10}$ values of colony forming units decreased from $1x10^5$ at 3 hpi to $1x10^3$ within 11 days. Further mutant studies with *X. campestris* pv. *vesicatoria* revealed that the epiphytic survival of *ΔhrpG* and *ΔhrpX* mutants is more compromised than that of mutants of the Hrp pilus protein *ΔhrpE* (Wengelnik *et al.*, 1996). The results obtained with other *Xanthomonas* spp. therefore implied that HrpG and HrpX are also capable of activating factors independent of the T3SS that rather contribute to the epiphytic phase than to infection itself. However, this appears not to be true for *Xtg*, since we could still detect nearly the same population densities of the *ΔhrpG* mutant compared to the wildtype *Xtg*29 isolate until 28 dpi. Our results therefore indicate that the *hrpG* gene is not primarily responsible for survival during the early infection stages but rather is responsible for symptom development (Fig. 4.7). However, when infecting *L. multiflorum* plants with *Xtg*, the leaf tissue is wounded by cutting with scissors. This procedure enables direct contact of the bacterial cells with the site or tissue of multiplication i.e. the xylem. Therefore, we hypothesize that due to the fact that *Xtg* cells gain direct access to the xylem, *Xtg* can make use of nutrients available inside the host throughout the infection process. However, symptom development appears to be dependent on the T3SS (or its induction) in

some way when comparing the symptoms caused by the *ΔhrpG* mutants with the symptoms caused by the wildtype strain (Fig. 4.6). This mechanism is, so far, not clarified and requires further experimental exploration. This would include, on one hand, mutants deficient of T3SS structural proteins and their comparison with the *ΔhrpG* mutants in order to draw conclusions concerning the importance of the T3SS. On the other hand, *ΔhrpG* mutants also deficient of another T3SS structural protein would clarify the role of *hrpG* in the infection process.

Nevertheless, another possibility why high bacterial populations densities are reached until 28 dpi, is that there may be alternative signalling mechanisms for the induction of *hrp* gene expression. Alternative *hrp* gene regulators belonging to the two-component signal transduction systems ColR/ColS were found in the pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) which causes black rot disease on cruciferous crops (Zhang *et al.*, 2008). The ColR/ColS two-component signal transduction system has originally been identified in the root-colonizing bacterium *Pseudomonas fluorescens*. *P. fluorescens* is used as biocontrol agent and the ColR/ColS has been identified to be a regulatory system involved in the capacity of *Pseudomonas fluorescens* to colonize plant roots. In this context, the ColS/ColR system was shown to act as a sensor and regulator. In the study with *Xcc*, the entire genome was screened for different ColR/ColS two-component regulatory systems. In total, three ColR/ColS sensor systems were found and three mutants deficient of one of these ColR/ColS sensor systems were generated. The outcome showed that in addition to HrpG and HrpX, one copy of this ColR/ColS system is capable of controlling the expression of the *hrpC* and *hrpE* operons. This study therefore suggested another two-component regulatory system as alternative or additional signalling pathway to HrpG/HrpX which could also be present in *Xtg* taking over the signalling mechanisms in place of *hrpG*.

Beside type III secretion activation, the two-component regulatory system HrpG/HrpX also has been shown to play a crucial role for other factors potentially associated with pathogenicity and host adaptation. These include type II secretion, the control of expression of lectins, and the biosynthesis of polyamines and phytohormones, such as ethylene and auxin (Wang *et al.*, 2008). Regulation of the type II secretion system (T2SS) has been demonstrated with the two polygalacturonases of *Xanthomonas campestris* pv. *campestris* PghAxc and PghBxc. Since HrpX-regulated promoters often carry such plant inducible promoter (PIP) boxes, and PghAxc and PghBxc both have been shown to contain a perfect PIP box, it has been concluded that type II secretion is also regulated by the HrpG/HrpX two-component regulatory system. Regulation of type II secretion by the *hrpG* gene has also been demonstrated for *X. axonopodis* pv. *citri* (*Xac*) strain NA-1 (Yamazaki *et al.*, 2008). Using

proteomic analyses, it has been demonstrated that *hrpG* regulates at least 11 genes associated with the T2SS. Since the T2SS is responsible for the secretion of toxins, extracellular enzymes, proteases, lipases and cell-wall degrading enzymes, it may also be concluded that secretion of the virulence factors secreted by means of the T2SS is defective by the *hrpG* mutation leading to a less rapid xylem colonization and symptom development.

In summary, it is concluded from the *hrpG* mutation experiment that *Xtg* depends on the *hrpG* gene for symptom development, however, in contrast to other *Xanthomonas* spp., *in planta* survival and multiplication are not affected by the *hrpG* mutation. Therefore, other regulatory components have to be investigated in order to fully understand the role of the T3SS and the *hrpG* gene for host colonization. Elucidating the presence of a homologous ColR/ColS system in *Xtg* and/or mutating this system may provide information concerning substitutability of the HrpG/HrpX system.

4.6 Acknowledgements

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5 Whole genome sequence analysis of *Xanthomonas translucens* pv. *graminis* with particular focus on the type III secretion system

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Unpublished

5.1 Introduction

Bacterial wilt caused by *Xanthomonas translucens* pv. *graminis* (*Xtg*) is a major disease of forage grasses such as Italian ryegrass (*Lolium multiflorum* Lam.). The disease seriously affects plant growth and reduces forage yield and quality (Egli *et al.*, 1975; Suter *et al.*, 2005). The pathogen invades the plant through wounded tissue and colonizes xylem vessels. Symptoms include wilting of the leaves beginning at the tip of the leaves progressing to the base leading to necrosis of entire plants. Breeding for resistance is the only practicable means of disease control, but due to population-based breeding schemes susceptible individuals often occur in advanced breeding germplasm. Identifying genes associated with the host-pathogen interaction represents a major objective in order to understand pathogenic interactions and to develop novel resistance breeding strategies based on plant genes and quantitative trait loci (QTL). The type III secretion system (T3SS) is a major virulence factor of most *Xanthomonas* spp. and has been reported to be responsible for the secretion of effector proteins that promote pathogen growth inside their hosts and suppress host resistance (Galan & Collmer, 1999). The existence and secretion of such effector proteins encoded in the genome of *Xtg* and the importance of a putative T3SS in *Xtg* has been a main subject of this project (chapter 4). Primers have been designed on the consensus sequence of published *Xanthomonas* spp. for a number of different genes encoding T3SS components in order to identify potential virulence factors in *Xtg*. However, despite high conservation among the sequences of the T3SS of published *Xanthomonas* spp., this approach has been shown to be only moderately effective for amplification and sequencing of virulence factors in *Xtg* (Müller-Hug, 2008). This may be due to the phylogeny of *Xanthomonas* spp. (see 1.2.2) based on 16S ribosomal RNA gene sequencing, indicating that *Xtg* is more distantly related to the other fully sequenced *Xanthomonas* spp. (Hauben *et al.*, 1997).

Whole genome analyses and comparative genomics are powerful tools to discover genetic features and virulence factors of related bacteria that have been acquired, modified, or lost during adaptation to their hosts (reviewed in Raskin *et al.*, 2006). This tool has also been especially useful for the identification of effector proteins and virulence factors resulting in a more comprehensive understanding of adaptation of pathogens to their hosts. This includes evolution of pathogenesis and other specific functions related to pathogenesis. To date, nine different genomes of *Xanthomonas* strains belonging to five different species have been fully sequenced and deposited in GenBank. Draft sequences of additional *Xanthomonas* strains belonging to four different species are available. The number of whole genome and draft sequences is increasing continuously with the improvement of existing and the development

of new sequencing technologies. Currently, there are whole-genome sequences available for three different *X. campestris* pv. *campestris* (*Xcc*) strains (i.e. ATCC33913, 8004 and B100), the vascular pathogen of cabbage and *Brassicaceae* including the model plant *Arabidopsis thaliana* (Da Silva *et al.*, 2002; Qian *et al.*, 2005; Vorhölter *et al.*, 2008).

Recently, the unique genome of the insect-vectored pathogen *Xanthomonas albilineans* causing leaf scald on sugarcane was made available (Pieretti *et al.*, 2009). *Xanthomonas albilineans* cannot survive in the environment and is strictly xylem-limited, living only in dead xylem cells or tracheary elements moving from host to host using insects as vectors (Rott & Davis, 2000). The genome of *Xanthomonas albilineans* is of particular interest, since it is most closely related to *Xtg* compared to all the other sequenced *Xanthomonas* spp. based on 16S ribosomal RNA sequencing (Hauben *et al.*, 1997). However, this study showed that *X. albilineans* does not possess *hrp* genes encoding a T3SS or any of the known T3SS secreted effectors. In addition, *X. albilineans* has a smaller genome when compared to the other sequenced *Xanthomonas* spp. and no genes encoding xanthan biosynthesis were found in *X. albilineans*. Unique for *X. albilineans* is also the production of the antibiotic and phytotoxic compounds albicidins. Albicidins are potent DNA gyrase inhibitors capable of blocking the religation of cleaved DNA intermediates during gyrase catalysis, resulting in lethal double-stranded DNA breaks (Hashimi *et al.*, 2007). Although the production of albicidins provides an advantage for survival of *X. albilineans*, increased mutation rates have been observed due to DNA repair errors of the polymerases which was hypothesized to be the cause for genome reduction. However, comparative phylogenetic analyses with *Xylella fastidiosa* (*Xf*), also a xylem-limited phytopathogen with a wide host range and no capability of albicidin production, revealed a convergent reductive genome evolution (Pieretti *et al.*, 2009). This rather indicated that a distinctive process had been responsible for the reductive genome evolution in *X. albilineans* and *Xf* during the descent from the progenitor of the *Xanthomonas* genus.

Further, sequence information is available for one strain of *X. axonopodis* pv. *citri* (*Xac* str. 306), the causal agent of citrus canker and which lives in the interveinal mesophyll apoplast (mesophyllic; Da Silva *et al.*, 2002); one strain of *X. campestris* pv. *vesicatoria* (*Xcv* str. 85-10), also a mesophyllic pathogen (chapter 1), that causes leaf spot on pepper and tomato (Thieme *et al.*, 2005); and three strains of *X. oryzae* pv. *oryzae* (*Xoo* strains KACC10331, MAFF311018 and PXO99A), the vascular pathogen of rice (Lee *et al.*, 2005; Ochiai *et al.*, 2005; Salzberg *et al.*, 2008). Unique for *Xoo* is the great genetic diversity among isolates that reflects rapid adaptation of the pathogen to the variety of host genotypes and

diverse environmental conditions where rice is grown (Hua *et al.*, 2007). Comparisons of the three sequenced *Xoo* isolates revealed major genomic rearrangements between the isolates, confirming that these genomes are highly plastic and rapidly evolving. Further, the presence of large numbers of insertion (IS) elements and type III secreted transcription activator-like effectors (TALEs) is another typical characteristic of *Xoo* isolates (Salzberg *et al.*, 2008).

An additional whole genome sequencing study has addressed the identification of genetic determinants of bacterial pathogenicity in *Ralstonia solanacearum* (*Rs*), a soil-borne root-colonizing pathogen that causes bacterial wilt on over 200 different plant host species (Salanoubat *et al.*, 2002). Although *Rs* belongs to the subdivision of β-proteobacteria, it has been shown to possess a *hrp* gene cluster encoding a T3SS belonging to the Hrp2 family (reviewed in Cornelis, 2006) similar to the T3SS of *Xanthomonas* spp. The whole genome of the *Rs* GMI1000 strain consists of two circular replica, a large one (3.7 Mbp) and a smaller one (2.1 Mbp). The *hrp* gene cluster is encoded on the smaller replicon which is referred to as megaplasmid and over 40 different type III secreted effectors have been identified that are distributed on both replica.

The aim of the present study was to sequence the whole genome of *Xtg* isolate 29 (Kölliker *et al.*, 2006) by means of 454 pyrosequencing (Margulies *et al.*, 2005) and to compare the genome sequence with the genomes of other *Xanthomonas* spp. Identifying genes in *Xtg* associated with pathogen virulence and comparing them to the known virulence factors such as the T3SS, effector proteins, EPS and LPS of other *Xanthomonas* spp. may allow for an effective analysis of the T3SS and effector proteins of *Xtg*. This process may enable a more profound understanding of host colonization strategies deployed by *Xtg* and may provide the basis for targeted *R*-gene identification in *L. multiflorum*.

5.2 Materials and Methods

5.2.1. Extraction of genomic DNA

Genomic DNA of isolate *Xtg*29 (Kölliker *et al.*, 2006) was extracted from 30 ml of bacterial culture grown at 28°C in Circle Grow broth (Molecular Probes) using a modified version of the CTAB method described in Ausubel *et al.*, (1987). In brief, after harvesting, bacterial cells were washed with Tris-HCl pH 8.0, 50 mM Na_2-EDTA, 150 mM NaCl-solution and 0.1% Tween80. After centrifugation, the pellet was resuspended in 8 ml TE buffer containing 0.5% SDS and 0.2 mg/ml proteinase K. After incubation for 1 hour at 50°C, 0.75 mg/ml RNAse A was added and incubated for another 30 min. at 37°C. After addition of 1.35 ml 5 M NaCl-solution, 1 ml CTAB/NaCl solution (10% CTAB, 0.7 M NaCl) was added and incubated for

10 min at 65°C. Then, an equal volume of chlorophorm/isoamyl alcohol (24:1) was added and mixed. After centrifugation, the supernatant was transferred to a fresh tube. An equal volume of phenol/chlorophorm/isoamyl alcohol (25:24:1) was added and mixed vigorously and after centrifugation, the supernatant was again transferred to a fresh tube. This step was repeated twice.

To precipitate the genomic DNA, 0.6 volumes of cold isopropanol stored at -20°C were added and mixed gently until the DNA precipitated. The precipitate was separated from the isopropanol with the hook of a sealed Pasteur pipette and washed in 70% ethanol. After two additional washes in 70% ethanol and 100% ethanol, the DNA was air dried for 5 min. Then, the DNA was dissolved in 100 µl of TE buffer over night.

5.2.2 Preparation of paired-end library

The genomic DNA was nebulized to obtain random fragments with an average size of 3kb. The GS Titanium Library Paired End Adaptors Kit (Roche, Mannheim, Germany) was employed to generate a 3K Paired End (PE) fragment library from the nebulized sample. After titration with a GS Titanium SV emPCR Kit (Lib-L) v2 (Roche), an emulsion PCR was carried out with the GS Titanium LV emPCR Kit (Lib-L) v2 (Roche) to obtain the sequencing samples that were analyzed using a GS Titanium Sequencing Kit XLR70t and the GS Titanium PicoTiterPlate Kit 70x75 (both from Roche) by means of a 454 Genome Sequencer FLX System (Roche). DNA Sequence read data resulting from the sequencing run were assembled using the Newbler software obtained from the manufacturer (454 Life Sciences, Roche Corporation).

5.2.3 Genome analysis and annotation

The finished genome data were annotated by using the GenDB software (Meyer *et al.*, 2003). Gene predictions were performed using the tools Prodigal, Critica and Glimmer. For the genome of *Xtg*29, the gene finder Prodigal has been shown to yield the most suitable prediction for coding sequences (CDS). Therefore, all the further analyses are based on the analysis using Prodigal and leading to sequence identifiers such as Xtg29_prodigal_XXXX. DNA and protein sequences were compared using BLASTn, BLASTx or BLASTp (Altschul *et al.*, 1990). Predicted *Xtg* amino acid sequences were compared using the identity matrices after CLUSTALW analyses in Bioedit (Hall, 1999).

5.2.4 Sequencing of gaps

The following four primers hrpX1 (GCTCAACAGCCTGAGCCGGG) hrpX2 (CGTCGGCCGCGAGCATCTGG), hrcT1 (CGATACCGTCAGGCTGCGCT), hrcT2 (GAATCAAGTCTGTATTTGCAG) were used to sequence the gap in the genome sequence between the *hrpX* and the *hrcT* gene. PCR reactions were performed using Phusion (Finnzymes, Espoo, Finland) DNA polymerase (see 4.3.3) and the resulting products were cut from the agarose gel (1%) using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Sequencing using Sanger sequencing technology was performed using BigDye v.1.1 and ABI3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA) as described in 4.3.3.

5.3 Results and Discussion

5.3.1 General features of the *Xanthomonas translucens pv. graminis* isolate 29 (*Xtg*29) genome

A draft sequence of the *Xtg*29 genome was obtained by employing a whole genome shotgun approach by means of 454 sequencing (Roche). For this purpose, a library of paired-end (PE) shotgun fragments was prepared from the genomic DNA. The fragment library was sequenced using the 454 Titanium technology. The sequencing run resulted in the generation of 905,328 individual sequences (reads), whereof 859,900 with in total 206,326,978 bases could be utilized successfully in the subsequent assembly step. Among these reads, 228,687 carried PE information. The PE data turned out to be very helpful when the assembly process encountered sequence gaps that could not be covered unambiguously with sequence reads. The reads were assembled using the Newbler software (Margulies *et al.*, 2005; Miller *et al.*, 2010). The obtained *Xtg*29 draft sequence consists of 908 contigs with gaps between the contigs. Therefore, all analyses may not reflect absolute values and may only serve as predictions. However, an average coverage of 45 fold resulting from the assembly with 454 reads indicates a high reliability of the contig consensus sequences.

From the assembled data, a G+C content of 68.6% was calculated. This G+C content is higher than in all the previously determined complete genomes of *Xcc*, *Xoo*, *Xac*, *Xcv*, and *X. albilinieans*, where the G+C content was well conserved at about 63% to 65%. The unusual high G+C content may be a factor that may have complicated the sequencing process. Another factor that may hinder the automated assembly is the presence of repetitive sequences. A statistical analysis of the contig coverage by sequence reads indicates the presence of such repeats in considerable number. There were 30 short contigs that were

calculated to be represented with five or more identical copies in the chromosome. All contigs but one, which showed no similarity to sequence database entries, encoded transposases or transposase fragments from IS elements. The most frequent repeat was similar to a 102 bp short fragment of the IS1480a transposase gene from *Xcc*. 290 copies in the *Xtg*29 genome were calculated solely for this repeat. In total, the copy number for the 30 most frequent repeats was 1904, but there were indications that in many cases two to four of these copies were associated in a conjoint genomic locus. Nevertheless, it becomes evident that the number of IS elements or IS element fragments in the genome of *Xtg*29 will develop into several hundred copies. High numbers of both complete and fragmentary IS elements have been observed for all *Xoo* genomes so far, as well as for *X. oryzae* pv. *oryzicola*. Hence, this finding relates *Xtg* to the rice pathogenic *Xanthomonas* strains, where the number of IS elements ranges from 251 complete to 714 fragments of IS elements. *Xoo* does not only contain the most but also the most diverse pool of IS elements (Salzberg *et al.,* 2008). Of the 19 known IS element families (Siguier *et al.,* 2006), eight families representing 28 distinct element types appear in *Xoo* genomes. In any case, these sequence repeats are an obstacle for automated assembly procedures. Thus in *Xtg*29, the presence of many IS element copies may be the main reason for the number of gaps that remained between the contigs in the automated assembly.

Among the 908 contigs obtained from the assembly, 132 that were shorter than 500 bases were excluded from subsequent analysis. From the remaining contigs, 654 could be arranged in 14 scaffolds. Within these scaffolds, the succession of the individual contigs is clear, and the size of the gap can be reliably predicted from the PE data. Overall, the scaffolds comprised 4,559,659 bases. By appending the remaining contigs that were longer than 500 bases, a chromosome size of at least 4,660,771 bp emerged. However, this size considers repetitive sequences only with a single copy. Short repeats may be completely unaccounted. The additional total sequence length reflecting all copies of the 30 most frequent repeats adds to 396,295 bp. This results in a predicted genome size of 5,057,066 bp. The assembled data were in line with a genome consisting of one single, circular chromosome (Fig. 5.1) and no plasmids. The genome size was comparable to the other xanthomonads deposited in public databases. While *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) and *X. axonopodis* pv. *citri* (*Xac*) carry four and two plasmids, respectively, no plasmids were found in the other seven fully sequenced *Xanthomonas* spp.

The predicted number of ribosomal RNA (rRNA) operons was 5 and the predicted number of transfer RNAs (tRNA) was 52. Interestingly, all the other sequenced *Xanthomonas*

spp. have only 2 rRNA operons and between 51 and 55 tRNAs. *X. translucens* pv. *graminis* contains a total of 3,931 predicted CDS which is comparable to those of the other fully sequenced *Xanthomonas* strains containing between 3,115 (for *X. albilineans*) and 5,083 (for *Xoo* PXO99A). However, considering that the genome of *X. albilineans* has experienced drastic genome reduction during evolution, this comparison may not be appropriate. When excluding the low number of CDS of the *X. albilineans* strain, the median of CDS is 4,487 indicating that *Xtg*29 has a rather low number of predicted CDS compared to the other *Xanthomonas* spp. However, due to the prediction of gaps of >200 bp between the contigs, the fully sequenced genome of *Xtg* may reveal additional CDS. Furthermore, the number of CDS predicted for the *Xoo* genomes is inflated by the huge number of IS elements within them. As each IS element encodes a complete or truncated transposase, a consideration of the correct number of IS elements will increase the number of CDS for *Xtg*29 analogously.

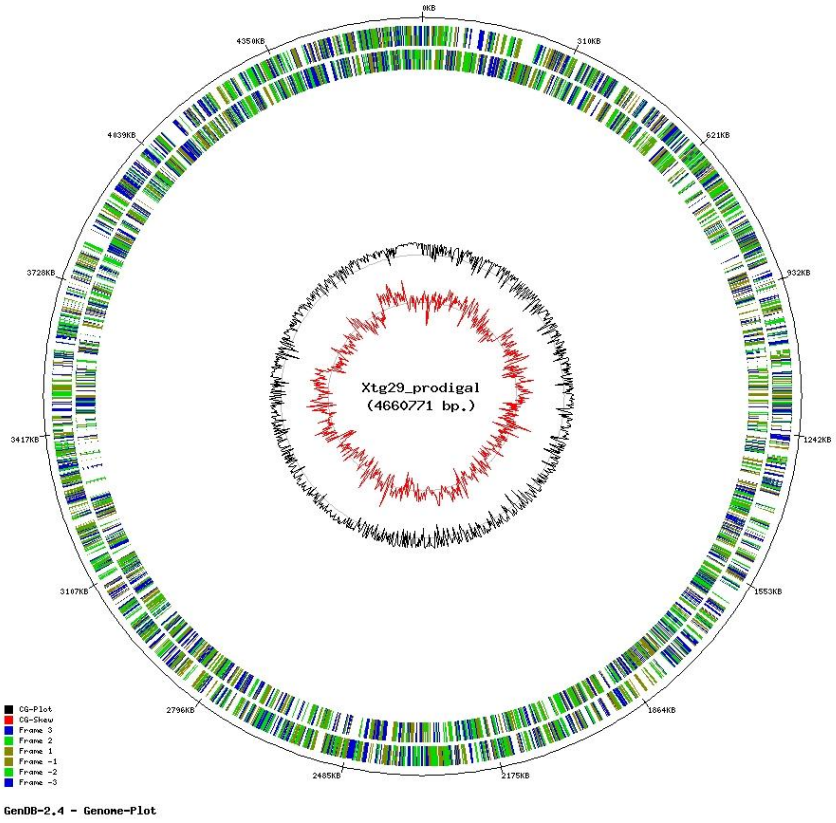

Figure 5.1 Circular plot of the *X. translucens* pv. *graminis* 29 draft genome. From the outer to the inner circle: circle 1: genomic positions in kilobases; circle 2: predicted CDS on the forward strand; circle 3: predicted CDS on the reverse strand. The black inner circle indicates the C+G-plot and the red circle indicates the G+C-skew.

5.3.2 The type III secretion system (T3SS) of *Xtg*29

The T3SS is a major virulence factor of *Xanthomonas* spp. and is responsible for the translocation of effector proteins which promote disease inside the host. In *Xtg*, a single *hrp* gene cluster encoding components of the T3SS was found to be localized on a gene cluster consisting of 20,764 bp and eleven *hrc* genes, eight *hrp* genes and three *hpa* genes (Table 5.2 and Fig. 5.2). In the original draft sequence, there were two gaps within the T3SS gene cluster (i.e. one gap between the genes encoding *hrpX* and *hrcT* and one between *hrpB4* and *hrcJ*). One of the gaps (between *hrpX* and *hrcT*) was closed by means of Sanger sequencing of four different PCR products. Alignment of the sequenced PCR products revealed a 815 bp long sequence between the two contigs and BLASTn search (http://blast.ncbi.nlm.nih.gov/) of the sequence revealed high nucleotide sequence similarity (88.3%) to two IS elements of *Xoo* (ISXoo11 and ISXoo12; Fig. 5.2).

The two genes *hrpG* and *hrpX* encoding the two response regulators of the T3SS were found to be localized within the *hrp* gene cluster in *Xtg*29 which is in contrast to the other sequenced *Xanthomonas* spp., where *hrpG* and *hrpX* are situated together and outside of the *hrp* gene cluster (Fig. 5.2). A similar architecture with the genes encoding *hrpG* and *hrpB* (which is homologous to *hrpX* of *Xanthomonas* spp.) within the T3SS has been reported for the *Ralstonia solanacearum* isolate GMI1000 for which the T3SS has been shown to be located on the megaplasmid pGMI1000MP (Salanoubat *et al.*, 2002; Fig. 5.2). Nevertheless, the protein sequences of the individual T3SS components appeared to be more similar to *Xanthomonas* spp. than to *Ralstonia solanacearum* pGMI1000 with only the highly conserved proteins HrcN and HrcV showing high sequence similarities (Fig. 5.2). The facts that in other *Xanthomonas* spp. the *hrpX* gene is located somewhere else in the genome and that there are two IS elements flanking the *hrpX* gene indicate that rearrangements in the T3SS architecture of *Xtg* may have occurred.

Average T3SS protein sequence identities derived from CLUSTALW analyses of the *Xtg*29 isolate and four other *Xanthomonas* spp. (i.e. Xcv: *Xanthomonas campestris* pv. *vesicatoria* str 85-10, Xac: *Xanthomonas axonopodis* pv. *citri* str. 306, Xoo: *Xanthomonas oryzae* pv. *oryzae* PXO99A) revealed that the proteins of the T3SS are less conserved between *Xtg*29 and each of the four isolates compared to conservation among the other *Xanthomonas* spp. Across the entire T3SS, the proteins sequence of HrpB7, HrpE, HpaA and HrpD6 are the least conserved between *Xtg*29 and the other four *Xanthomonas* spp. used for this comparison. This corresponds to the average identity of the analyzed *Xanthomonas* spp. for the HrpE and HpaA proteins; however HrpB7 and HrpD6 appear to be more conserved

among the other *Xanthomonas* spp. compared to HrpE and HpaA. On the other hand, the most conserved proteins are HrcR and HrcN with average identities of 77.38% and 73.63% average identities across all *Xanthomonas* spp. used for the comparison. This also corresponds to the average identities of 97.48% for HrcR and 93.87% for HrcN between the other *Xanthomonas* spp. without *Xtg*29. This analysis suggests that the T3SS of *Xtg*29 has, although it appears to be related to the other *Xanthomonas* spp., very distinct characteristics and may therefore have distinct functions.

Table 5.2 Protein sequence comparisons based on phylogeny of type III secretion system components of *Xanthomonas translucens* pv. *graminis* 29 to other *Xanthomonas* spp. i.e. Xcv: *Xanthomonas campestris* pv. *vesicatoria* B100, Xac: *Xanthomonas axonopodis* pv. *citri* str. 306, Xoo: *Xanthomonas oryzae* pv. *oryzae* PXO99A and average protein sequence identity of the four presented *Xanthomonas* spp.

	Identity of protein sequences of *Xtg*29 compared to other *Xanthomonas* spp.				
Protein name	**Xcv**	**Xac**	**Xoo**	**Xcc**	**Average identity among *Xanthomonas* spp. (without *Xtg*29)**
HrcC	54.90%	55.30%	54.70%	54.00%	89.10%
HrpX	63.80%	63.80%	61.10%	63.80%	92.07%
HrcT	60.40%	59.30%	59.30%	59.70%	91.91%
HrpB7	26.40%	27.00%	27.00%	27.00%	79.13%
HrcN	73.30%	73.50%	73.50%	74.20%	93.87%
HrcL	42.20%	40.90%	41.80%	43.40%	85.73%
HrpB4[1]	[1]	[1]	[1]	[1]	77.57%
HrcJ	60.30%	59.50%	57.70%	60.60%	88.43%
HrpB2	36.90%	39.20%	37.60%	37.60%	84.45%
HrpB1	40.50%	41.10%	41.10%	45.00%	86.50%
HrcU	52.30%	52.60%	53.40%	54.90%	87.26%
HrcV	67.10%	66.60%	65.40%	67.80%	94.41%
HpaP	25.00%	25.00%	24.30%	22.60%	63.10%
HrcQ	28.20%	27.60%	26.70%	30.90%	74.58%
HrcR	77.60%	77.60%	76.20%	78.10%	97.48%
HrcS	67.40%	67.40%	50.00%	67.40%	86.38%
HpaA	25.00%	25.00%	24.30%	22.60%	69.02%
HrcD	32.00%	32.70%	23.80%	31.40%	73.93%
HrpD6	26.10%	28.90%	30.10%	30.10%	79.98%
HrpE	23.70%	24.70%	17.50%	21.60%	53.60%
HpaB	47.70%	47.70%	20.50%	45.90%	63.60%
HrpG	51.80%	52.20%	51.80%	52.60%	80.93%

[1]Comparison not possible, coding sequence still contains a gap and has not been completely sequenced in *Xtg*29.

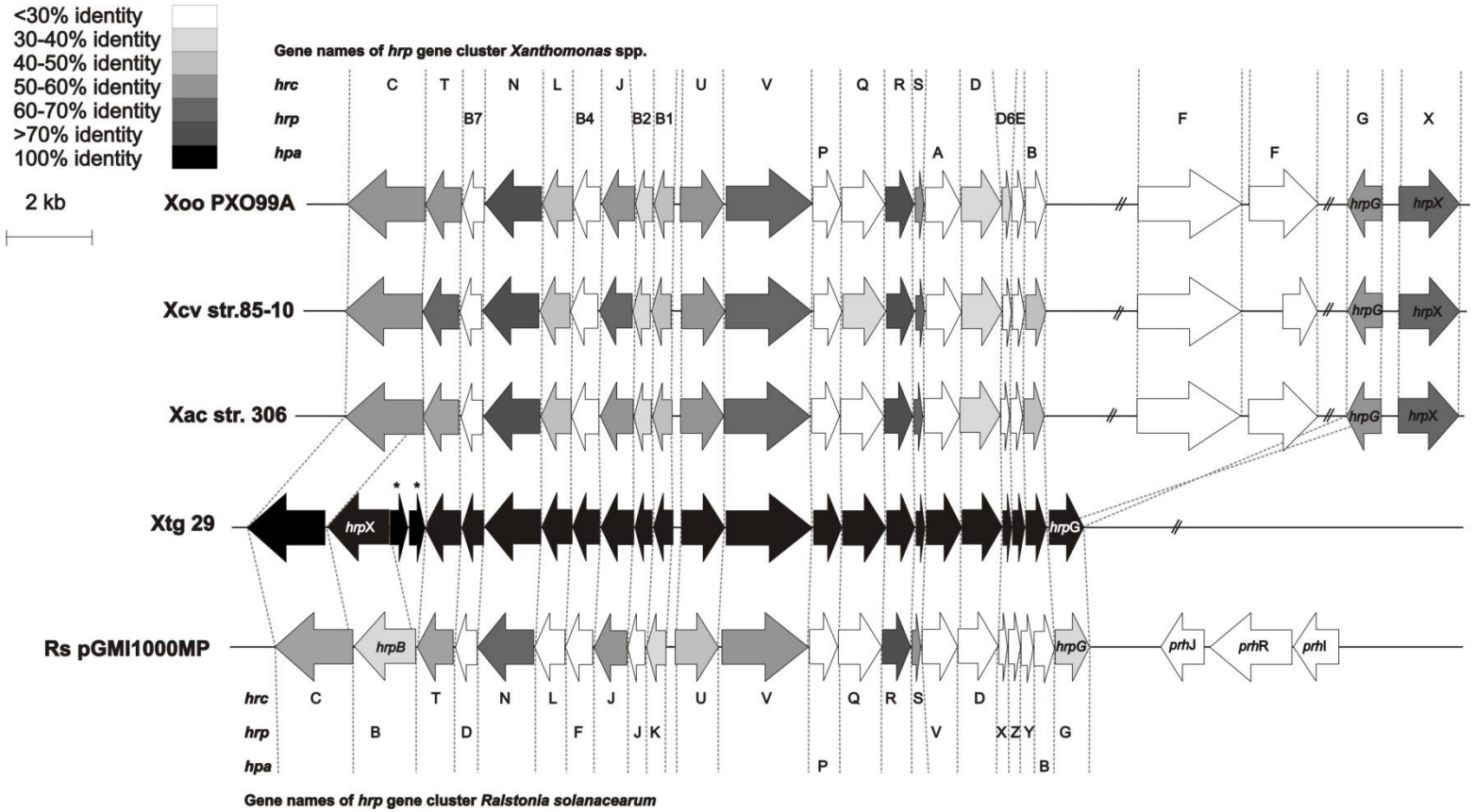

Figure 5.2 Protein sequence identity of the *hrp* proteins from different sequenced *Xanthomonas* spp. and *Ralstonia solanacearum* GMI1000 compared with *X. translucens* pv. *graminis hrp* genes. Genes of the following strains are shown: Xoo: *X. oryzae* pv. *oryzae* PXO99A (Salzberg *et al.*, 2008), Xcv: *X. campestris* pv. *vesicatoria* strain str 85-10 (Thieme *et al.*, 2005), Xac: *X. axonopodis* pv. *citri* strain 306 (Da Silva *et al.*, 2002), and Rs: *Ralstonia solanacearum* GMI1000 Megaplasmid pGM1000MP (Salanoubat *et al.*, 2002). Arrows indicate the sizes, positions, and orientations of the *hrp*, *hrc*, and *hpa* genes. The two genes indicated with asterisks (*) were shown to be similar to ISXoo11 and ISXoo12 transposases. The identity of each protein sequence with its homolog in *X. translucens* pv. *graminis* is presented by use of a black/gray color scale.

5.3.3 Predicted genes encoding effector proteins secreted through the T3SS

The T3SS of Gram-negative phytopathogens typically translocates a cocktail of different effector proteins into the plant cell (Furutani *et al.*, 2009; Roden *et al.*, 2004; Thieme *et al.*, 2005; White *et al.*, 2009). A number of different type III secreted effectors with different molecular functions from *Xanthomonas* spp. and *Ralstonia* spp. have been described (reviewed in Kay & Bonas, 2009) and typically one bacterial isolate carries genes encoding between 20 and 30 different effectors. In *Xtg*29, genes encoding 22 proteins homologous to type III secreted effectors have been identified, so far (Table 5.3). This indicates that the variety of type III secreted effectors may correspond to the typical number of translocated effector proteins. However, there may also be additional, so far uncharacterized effector proteins in *Xtg*29. The functions of some type III secreted effectors for which encoding genes were found in *Xtg*29 have been extensively studied, while others revealed BLASTx results indicating some similarity to effector proteins of other phytopathogenic bacteria (Table 5.3).
The genes encoding type III secreted effectors included two copies of the *xopD* gene encoding small ubiquitin-like modifier (SUMO) protein cysteases of the C48 family. XopD evidently cleaves SUMO precursors and removes SUMOs from plant proteins (Hotson *et al.*, 2003). In contrast to ubiquitination, SUMOylation of eukaryotic proteins stabilizes them by covalently linked SUMO. Therefore, in plants, SUMOylation and deSUMOylation regulate a number of crucial processes, such as abiotic stress responses, pathogen defence, and flower induction (Novatchkova *et al.*, 2004). Since XopD is localized in the nucleus and binds unspecifically to DNA via a putative helix-loop-helix domain, it could target components of the plant transcription machinery. Further, two ethylene response factor-associated amphiphilic repression (EAR) motifs were detected within XopD. EAR motifs are found in plant transcription factors that repress defence and stress responses (Kim *et al.*, 2008).

Two proteins that are similar to AvrRxv effectors described in detail for *Xcv* were found in *Xtg29*. These two candidate effectors show significantly lower G+C contents (53% and 48%) which usually is indicative for acquisition by horizontal gene transfer (Dobrindt *et al.*, 2004). Acquisition by horizontal gene transfer is corroborated by the fact that one of the putative AvrRxv effectors is located in close vicinity of two different transposases. AvrRxv (or XopJ) is a member of a family of pathogen effectors that is also found in mammalian pathogens, for example YopJ and YopP from *Yersinia* spp., AvrA from *Salmonella*, and VopP from *Vibrio parahemeolyticus*. However, the AvrRxv/XopJ effector group may cover members with different enzymatic activities and target multiple host physiological pathways (Mukherjee *et al.*, 2007). In addition to AvrRxv, there are three other AvrRxv family

members present in *Xcv*, two genes with similarity to *avrPphE* originally identified in *Pseudomonas syringae* pv. *phaseolicola* by its ability to trigger R2 disease-resistance function in some bean cultivars (Mansfield *et al.*, 1994).

A homologous gene encoding the AvrBs2 effector has been identified in *Xtg*29. Genes encoding the AvrBs2 effector have been shown to be conserved among all the so far sequenced *Xanthomonas* spp. (reviewed in Büttner & Bonas, 2010). AvrBs2 encodes a putative glycerolphosphoryl diester phosphodiesterase with strong homology to the C-terminal entity of the agrocinopine synthase (ACS) of *Agrobacterium tumefaciens*. β-1,2-glucans may be a possible enzymatic target for AvrBs2. Cyclic β -1,2-glucans *in Agrobacterium, Rhizobium*, and *Xanthomonas* species have been implicated in both osmotic adaptation and plant host signalling (Swords *et al.*, 1996). Since a Δ*avrBs2* mutant was reduced in growth, the modified glucan has been proposed to promote *Xanthomonas* adaptation to the plant intercellular space. Nevertheless, despite conservation in many *Xanthomonas* spp., experimental evidence that translocation of AvrBs2 contributes to virulence and multiplication of the bacteria *in planta*, symptom development and epiphytic survival (Gürlebeck *et al.*, 2006; Mudgett *et al.*, 2000) has only been provided for *Xcv*.

Genes encoding effectors belonging to the highly conserved *avrBs3/pth* family also called transcription activator-like effectors (TALEs) present in many *Xanthomonas* spp. and *Ralstonia solanacearum*, were not found in the genome of *Xtg*29. While different isolates of *Xoo* have been shown to contain 19 genes alone for TALEs (Ochiai *et al.*, 2005; Salzberg *et al.*, 2008), complete absence of TALEs has previously also been reported for all sequenced *Xcc* isolates and the sequenced isolate of *Xcv* str. 85-10 (Thieme *et al.*, 2005).

Table 5.3 Type III secreted effectors and predicted effectors found in the genome of *Xanthomonas translucens* pv. *graminis* and putative functions or homologs from other pathogens using a type III secretion system (e.g. *Pseudomonas syringae* or *Yersinia* spp.).

Gene name	Sequence name	Function/ family/homology to *Pseudomonas* effectors	Length [bp]	Reference
avrBs2	Xtg29_prodigal_1815	putative glycerophosphoryl-diester phosphodiesterase	2148	Swords *et al.,* (1996)
avrRxv	Xtg29_prodigal_906	YopJ/AvrRxv family, putative cysteine protease	351	Whalen *et al.,* (1993)
avrRxv	Xtg29_prodigal_1040	YopJ/AvrRxv family, putative cysteine protease	1698	Whalen *et al.,* (1993)
xopB	Xtg29_prodigal_3309	homology to HopD1 [*P. syringae* pv. *tomato*]	1839	Nöel *et al.,* (2001)
xopD	Xtg29_prodigal_2359	SUMO protein cystease; C48 family	1722	Nöel *et al.,* (2002)
xopD	Xtg29_prodigal_2361	SUMO protein cystease; C48 family	1836	Nöel *et al.,* (2002)
xopE1	Xtg29_prodigal_1847	homology to *avrPphE*/HopX, predicted transglutaminase	1089	Thieme *et al.,* (2007)
xopF1	Xtg29_prodigal_2570	unknown function	2058	Roden *et al.,* (2004)
xopF2	Xtg29_prodigal_2237	unknown function	855	Roden *et al.,* (2004)
xopN	Xtg29_prodigal_017	homology to HopAU1 [*P. syringae*], α-helical ARM/HEATS repeats	2133	Kim *et al.,* (2009)
xopP	Xtg29_prodigal_2591	unknown function	1860	Roden *et al.,* (2004)
xopQ	Xtg29_prodigal_1645	HopQ1-1 family protein, putative inosine-uridine nucleosidee N-ribohydrolase	1407	Roden *et al.,* (2004)
xopX	Xtg29_prodigal_785	homology to HopAE1 [*P. syringae*]	1893	Metz *et al.,* (2005)
	Xtg29_prodigal_458	homology to HopH1 [*P. syringae*]	210	Wei *et al.,* (2007)
	Xtg29_prodigal_459	homology to HopH1 [*P. syringae*]	531	Wei *et al.,* (2007)
	Xtg29_prodigal_2027	homology to HopK1 [*P. syringae*]	813	Wei *et al.,* (2007)
	Xtg29_prodigal_251	Glycerophosphodiester phosphodiesterase	1017	
	Xtg29_prodigal_292	homology to *avrPphE*/HopX	990	
	Xtg29_prodigal_808	homology to HopX1	1131	Wei *et al.,* (2007)
	Xtg29_prodigal_95	homology to HopR	5181	Wei *et al.,* (2007)
	Xtg29_prodigal_1144	homology to AvrXv3/ HopAF1	189	Astua-Monge *et al.,* (2000)
	Xtg29_prodigal_3311	putative type III effector protein [*R. solanacearum*]	798	

5.3.4 Type I secretion system (T1SS) and *rax* gene homologs of *Xtg*

Secretion of the avirulence protein AvrXa21 requires the presence of the three *rax* (required for AvrXa21) genes *raxA* (encoding a membrane fusion protein), *raxB* (encoding an ABC transporter), and *raxC* (encoding an outer-membrane protein) that encode components of a

type one secretion system (T1SS; Lee *et al.*, 2006). Activity of the AvrXa21 protein further requires expression of the sulfotransferase *raxST* for sulfurylation. Expression induction of the *rax* genes depends on a two-component regulatory system *raxH*/*raxR* (Lee *et al.*, 2008), and the sulfur metabolism enzymes *raxP* and *raxQ*. It has been demonstrated with mutants deficient of the *hrpC* gene that AvrXa21 activity is independent of the T3SS and only depends on the functioning T1SS described above. The two-component regulatory system *raxH*/*raxR* responds to *Xoo* cell population density, and therefore the AvrXa21 pathogen-associated molecule has been proposed to be involved in quorum sensing which most likely is also conserved in other *Xanthomonas* spp. (Lee *et al.*, 2006).

The genes required for AvrXa21 activity (*rax* genes) have been studied in detail for the *Xoo* strain MAFF311018 (Ochiai *et al.*, 2005) and appropriate annotations have been included in the whole genome sequence of this *Xoo* strain. Therefore, comparative analyses of *rax* gene homologs were performed with this *Xoo* MAFF311018 strain. The EDGAR tool is an efficient software framework for the comparative analysis of prokaryotic genomes (Blom *et al.*, 2009). It allows a classification of genes as core genes or singletons and enables biological insights into the differential gene content of related genomes. In addition, it visualizes evolutionary relationships like synteny plots or Venn diagrams. The EDGAR tool was used to identify the core genome of *Xoo* MAFF311018 and *Xtg*29 and revealed homologous genes to the *rax* genes (Table 5.4).

Table 5.4 Homologs of genes required for AvrXa21 activity (*rax* genes) of *Xanthomonas translucens* pv. *graminis* compared to *Xanthomonas oryzae* pv. *oryzae* MAFF311018 with EDGAR.

Gene name	Sequence name	Protein family	Length [bp]	Reference
raxA	Xtg29_prodigal_608	membrane fusion protein	2169	Lee *et al.*, (2006)
raxB	Xtg29_prodigal_606	ABC transporter	822	Lee *et al.*, (2006)
raxC	Xtg29_prodigal_1389	outer-membrane protein	1365	Lee *et al.*, (2006)
raxST	Xtg29_prodigal_605	sulfotransferase	804	Lee *et al.*, (2006)
raxR	Xtg29_prodigal_615	response regulator	702	Lee *et al.*, (2006)
raxH	Xtg29_prodigal_616	histidine kinase	1293	Lee *et al.*, (2006)
raxP	Xtg29_prodigal_3309	sulfate adenyltransferase subunit 1	930	Lee *et al.*, (2008)
raxQ	Xtg29_prodigal_2359	sulfate adenyltransferase subunit 2	1983	Lee *et al.*, (2008)

Table 5.4 demonstrates that all components for secretion of a potential homologous avirulence factor to AvrXa21 are present in *Xtg*. However, whether such a molecule really is secreted remains to be elucidated. Numerous other genes with functions related to type I secretion of toxins (bacteriocins), lipases and proteins or encoding components of T1SS such

as ATP-binding cassette (ABC) protein transporters, membrane fusion proteins (MFP) and outer membrane proteins were found in *Xtg*29.

5.3.5 Genes encoding other virulence factors of *Xtg*

As it is the case for all sequenced *Xanthomonas* spp. (except for *X. albilineans*), the *Xtg*29 draft genome carries all genes responsible for xanthan biosynthesis organized in a cluster containing the genes *gumB* to *gumM* and covering a region of 13,043 bp. The synthesis of extracellular degrading enzymes and exopolysaccharides (EPS) is transcriptionally regulated by products of the regulation of pathogenicity (*rpf*) gene cluster. Mutations of the *rpfF*, *rpfG* or *rpfC* gene have been shown to lead to decreased levels of EPS and extracellular enzyme production and altered biofilm formation. Further, it was demonstrated in *Xcc* that *rpf*-dependent regulation is required to revert stomatal closure activated after pathogen attack as part of plant defense response (Gudesblat *et al.*, 2009). In *Xcc*, when the *rpfI* gene was inactivated, expression levels of proteases were reduced indicating that *rpfI* is responsible for tissue degeneration during *Xcc* infection (Dow *et al.*, 2000). However, similar to *Xac* and *Xf*, *Xtg*29 lacks the genes *rpfH* and *rpfI*. Besides the *rpf* gene cluster, three genes encoding proteins for xanthomonadin biosynthesis and export (Xtg29_prodigal_1704, Xtg29_prodigal_1706 and Xtg29_prodigal_1707) and three genes encoding lipopolysaccharides (LPS), i.e. *wxcI*, *wxcJ*, *wxcX* were discovered in the *Xtg*29 genome.

A *xps* gene cluster encoding components of a type II secretion system was found in *Xtg*29. The *xps* gene cluster covers a region of 11,732 bp and consisted of the genes *xpsEFGHIJKLMD*. However, the gene encoding *xpsO* typically present in close vicinity to the genes encoding the T2SS of *Xcc* was not found. On the other hand, another copy of the genes encoding *xpsKL* and an additional *xps* gene (*xpsN*) were discovered outside of the *xps* gene cluster.

In contrast to *Xcv*, where two different types of putative T4SS were found (Thieme *et al.*, 2005), no T4SS was identified by automatic annotation of the genome of *Xtg*29. The only genes with T4SS-related annotations were three copies of a VirJ-like protein. VirJ is a soluble periplasmic protein and it has been shown to exhibit specific interactions with both the transport apparatus and type IV secretion substrates (effectors) during *Agrobacterium tumefaciens* infection (Pantoja *et al.*, 2002). However, no T4SS or other type IV secreted effectors were identified and whether VirJ could also interact with type III secreted substrates or which significance VirJ has for pathogenicity of *Xtg* might be a very interesting objective for further mutant studies with *Xtg*.

5.4 Conclusions

Despite a number of similarities to the other sequenced *Xanthomonas* spp. (e.g. the *gum* gene cluster, EPS production and conserved *hrp* genes), the isolate *Xtg*29 was found to be different from the other sequenced *Xanthomonas* spp. in terms of T3SS architecture (Fig. 5.2) and protein sequence identity (Table 5.2). A set of homologous genes encoding type III secreted effectors that certainly is important for the interaction of *Xtg* with *L. multiflorum* was found. These effectors may be specific for the host-pathogen interaction and may also determine the host range of *Xtg*. Nevertheless, whether these homologs are functional and/or race-specific for the isolate *Xtg*29 or are also found in other *Xtg* isolates will require further experimental data. From the data obtained so far, it can be speculated that most likely not only virulence factors and symptoms may be distinct for the *L. multiflorum-Xtg* interaction, but also that resistance mechanisms may differ from well characterized systems such as rice-*Xoo* and pepper-*Xcv*. Similar to the host range of *Ralstonia solanacearum* which includes over 200 different host species, *Xtg* also has a broad host range among forage grasses. For this reason and due to similar T3SS gene clusters, we may have to rely on different phytopathogenic systems such as *Ralstonia solanacearum* for targeted resistance gene identification and to understand in more detail the interaction of forage grasses with *Xtg* in the future.

6 General discussion

6.1 Introduction

Understanding the way pathogens interact with their host plants has been a major goal in the field of plant pathology for many decades. Particularly, the identification of factors that contribute to the host-pathogen interaction and define host-specificity has been the subject of intensive research. The application of genomic approaches combined with transcriptome analyses enables the identification of genes and genomic regions that affect host resistance and pathogen virulence. The overall aim of this thesis was to understand in more detail the interaction of *Xanthomonas translucens* pv. *graminis* (*Xtg*), which causes bacterial wilt on forage grasses, with Italian ryegrass (*Lolium multiflorum*), a major forage grass of temperate regions. More specific, this thesis reports on the elucidation of race-specificity in the *L. multiflorum-Xtg* interaction (chapter 2), the analyses of candidate genes potentially involved in the control of bacterial wilt resistance in *L. multiflorum* (chapters 3 and appendix), and preliminary studies to understand bacterial colonization strategies of *Xtg* (chapters 4 and 5). The data presented in this thesis provides a more profound understanding of *L. multiflorum* resistance mechanisms and bacterial virulence factors of *Xtg.* The overall analyses revealed that *Xtg* resistance most likely is conferred by different resistance mechanisms and is not race-specific. Transcriptome analyses revealed a number of interesting candidate genes for functional marker development. Based on this knowledge, tools may be developed to enhance breeding resistant *L. multiflorum* cultivars by means of marker assisted selection (MAS).

6.2 Genetic control of bacterial wilt resistance in *L. multiflorum*

In forage production, the use of resistant cultivars is the most desirable method to prevent economic losses caused by pathogen infection. Therefore, many approaches and techniques have been developed for the phenotypic and genetic characterization of disease resistance in plants. In ryegrass breeding programs, phenotypic screening for resistance is the most widely used method of parental selection (reviewed in Brummer, 1999; Dracatos *et al.*, 2010; Kimbeng, 1999). Breeding of *Xtg* resistant *L. multiflorum* cultivars is based on artificial seedling inoculation and recurrent phenotypic screening of resistant parents, which are subsequently used for random mating (reviewed in Humphreys *et al.*, 2010). This approach has led to *L. multiflorum* cultivars with improved resistance to *Xtg*; however, susceptible individuals often occur in advanced breeding germplasm and further breeding progress with phenotypic selection is limited due to the obligate out-breeding reproduction mode (Michel, 2001). Therefore, molecular biology techniques for quantitative trait loci (QTL) analyses and molecular marker-trait associations are used to identify genomic regions that contribute to

host resistance and to identify the inheritance of complex traits. In *L. multiflorum*, *Xtg* resistance has been shown to be controlled by one major QTL on linkage group (LG) 4 and several minor QTL indicating qualitative *Xtg* resistance (Studer *et al.*, 2006) due to frequent association of major QTL with qualitative resistance (Mutlu *et al.*, 2005; Verdier *et al.*, 2004; Yang & Francis, 2005). However, the genes and genetic networks underlying these identified QTL remain unknown using solely QTL mapping and marker-trait associations. Therefore, transcriptome analyses and sequence-based approaches may help to identify candidate genes involved in disease resistance. This approach may lead to the identification of functionally favorable alleles which can be used for marker-assisted selection.

6.2.1 Qualitative or quantitative resistance?

Detailed knowledge on the inheritance mode of resistance is indispensible for breeding resistant forage grass cultivars. Qualitative resistance is conferred by single genes which may provide complete protection against a specific pathogen race or isolate. Single resistance (*R*)-genes conferring qualitative resistance are widely used in cereal breeding programs but are prone to breakdown due to changes in pathogen race structure. Thus, qualitative resistance is considered to be less durable compared to quantitative resistance which is conferred by several genes. However, quantitative resistance is difficult to maintain stably in a population due to the out-breeding reproduction mode of forage grasses. In *L. perenne* and *L. multiflorum*, both qualitative and quantitative resistance against *Puccinia coronata* f.sp. *lolii* races have been identified (Dumsday *et al.*, 2003; Wilkins, 1975). In this case, the single major *R*-genes (qualitative resistance) have been shown to be less effective compared to multiple minor genes for the maintenance of durable resistance (Wilkins, 1978). Alternatively, there are a few single *R*-genes that confer resistance to a wide range of different pathogen strains and still have been shown to be durable. These include, for example, the *Xoo* resistance gene *Xa21* (Song *et al.*, 1995) and the powdery mildew resistance gene *mlo* of barley (Büschges *et al.*, 1997). However, the *Xa21*-mediated resistance is race-specific (Wang *et al.*, 1996), whereas in the case of *mlo*, race-specificity is absent rather indicating non-host resistance mechanisms (Humphry *et al.*, 2006).

Qualitative *Xtg* resistance in *L. multiflorum* was proposed in Studer *et al.*, (2006) based on the identification of a major QTL on LG 4 explaining between 43 and 84% of the total phenotypic variance for *Xtg* resistance. Phenotypic traits that result in the identification of a major QTL have often been shown to be associated with one or only a few major *R*-genes (Mutlu *et al.*, 2005; Mutlu *et al.*, 2006; Verdier *et al.*, 2004; Yang & Francis, 2005). However, the existence of qualitative resistance has not been clarified in detail for the *L. multiflorum*-

Xtg interaction. The phenotypic resistance screen presented in this thesis (chapter 2) revealed that bacterial wilt resistance of a given plant genotype is effective against a broad range of *Xtg* isolates with significantly different virulence. This indicated that no race-specific resistance exists in the *L. multiflorum-Xtg* interaction. However, race-specificity does not only depend on the inheritance of *L. multiflorum* resistance but also on virulence mechanisms of the pathogen. Therefore, presuming that the used *Xtg* isolates all use similar virulence mechanisms (e.g. avirulence products like type III secretion system (T3SS) effector proteins, see 6.3.3), it cannot be excluded that *R*-genes exist which are effective against all tested *Xtg* isolates. Consistent with this, it has been shown that *Xtg* isolates exhibit a high level of genetic similarity (86%; Kölliker *et al.*, 2006) which is in contrast to systems with clearly defined race-specific interactions e.g. *Xoo*-rice interaction (Hua *et al.*, 2007). In addition, the broad host range of the *Xtg* pathovar and the fact that *L. multiflorum* is often grown in mixtures with other grass species that are potential hosts of *Xtg* could also explain why *Xtg* races do not evolve rapidly. In order to test this hypothesis, race-specificity of other *X. translucens* pathovars that are restricted to one plant species may be used for race-specificity elucidation. Alternatively, an explanation for the absence of race-specificity is the fact that the *Xtg* isolates used in this thesis might not have captured the entire spectrum of genetic diversity of *Xtg* since they were exclusively sampled from pastures and meadows in Switzerland (Kölliker *et al.*, 2006). To gain a more profound overview concerning *Xtg* genetic diversity and race evolution, it would be interesting to sample additional *Xtg* isolates originating from other countries where bacterial wilt on forage grass has been reported and re-examine the level of genetic diversity.

Using marker-trait associations, *Xtg* resistance was found to be significantly correlated with the presence of one simple sequence repeat (SSR) marker on LG 5 in the same *L. multiflorum* genotypes. In contrast to the QTL identified on LG 4 (Studer *et al.*, 2006), this thesis supports the hypothesis that multiple loci of *Xtg* resistance in *L. multiflorum* exist. Supportive for the hypothesis of absence of qualitative *Xtg* resistance is also the absence of major transcriptional changes resulting from the hypersensitive response (HR; Bozso *et al.*, 2009) that typically occurs within 24 hours post inoculation (hpi; Scheideler *et al.*, 2002) which has been investigated by transcriptome analyses of a resistant *L. multiflorum* genotype (chapter 3; Rechsteiner *et al.*, 2006). Since the HR in plants is often the consequence of recognition of T3SS effectors (reviewed in Hogenhout *et al.*, 2009), this observation questions recognition of avirulence products (such as AvrRxv and AvrBs2, see 6.3.3) or T3SS effectors (see 6.3.4). Taken together, resistance mechanisms against *Xtg* appear to be very

complex and a clear qualitative resistance seems not to exist, rendering the identification of *R*-genes and breeding resistant *L. multiflorum* cultivars more difficult. Nevertheless, analyzing *Xtg* virulence mechanisms and T3SS effector proteins may represent a promising strategy to target in order to better understand *Xtg* resistance mechanisms in *L. multiflorum.*

6.2.2 Identification of candidate genes potentially involved in *Xtg* resistance

In addition to the phenotypic exploration of the inheritance of bacterial wilt resistance and molecular marker-trait associations, experiments using transcriptomics provide crucial insights into gene functions and/or global transcript levels. These insights can support the discovery of genes putatively involved in resistance to biotic stress and the identification of co-regulated genes which represent part of a regulatory network. Once candidate genes are identified by transcriptome analyses and they have been verified to control the trait of interest, the genes can be used for functional marker development. Functional markers are markers usually based on single nucleotide polymorphism (SNP) within genes controlling traits of interest which may be used for marker-assisted selection (MAS) during diagnosis of traits of interest in various breeding schemes (Andersen & Lübberstedt, 2003; Collard & Mackill, 2008; Francia *et al.*, 2005). The advantage of such markers is that they are not affected by the environment. Thus, selection can be performed at any stage of plant development and independent from the tissue (Francia *et al.*, 2005). Functional markers are preferred to anonymous markers such as amplified fragment length polymorphism (AFLP) or SSR markers because they are strictly coupled to the trait. Candidate genes for functional marker development have been identified in functional genomics studies and have been verified in marker-trait association studies for numerous traits (Flint-Garcia *et al.*, 2003; Rafalski, 2002). Further, functional MAS has been useful for introgression of three different *Xoo* resistance genes (*xa5*, *xa13* and *Xa21*) into highly *Xoo* susceptible but high-yielding rice genotypes (Perumalsamy *et al.*, 2010). This example demonstrates the potential of MAS to select for phenotypically superior plant genotypes with high levels of resistance.

6.2.2.1 Microarray detection

Transcriptome analysis using microarrays is a potential tool to discover candidate genes involved in biotic resistance and can reveal crucial insights into affected pathways occurring during defense responses (reviewed in Wan *et al.*, 2002; Wise *et al.*, 2007). However, there are certain restrictions to *R*-gene identification using transcriptome analyses. Limitations imposed to transcriptome analyses using the microarray technology include on one hand technical aspects such as high background levels and cross-hybridization with related

expressed genes or insufficient saturation of hybridization signals (Okoniewski & Miller, 2006). On the other hand, the probability of transcriptome studies to discover major *R*-genes may be limited when the *R*-gene itself is not represented on the microarray and/or cross-species hybridization to the *L. perenne* microarray may allow for the detection of a species-specific *R*-gene of *L. multiflorum*.

Another constraint includes the inability to detect constitutively expressed *R*-genes by comparing *Xtg* infected with control-treated samples. Constitutive expression has been shown for several *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) resistance genes in rice such as *xa5* (Jiang *et al.*, 2006b), *Xa21* (Century *et al.*, 1999) and *Xa26* (Sun *et al.*, 2004), or the leaf rust resistance gene *Lr19* of wheat (Gennaro *et al.*, 2009). The example of *xa13*-mediated resistance even demonstrates that expressional non-reaction of *xa13* to *Xoo* infection is the key factor for *xa13*-mediated resistance (Yuan *et al.*, 2009).

In order to increase the probability of capturing susceptibility genes such as *Xa13* (see 6.2.3.4) and constitutively expressed *R*-genes, transcriptome analyses comparing different *L. multiflorum* genotypes differing in bacterial wilt resistance was performed (chapter 3). Presuming similar transcriptomes of both of the used *L. multiflorum* genotypes as it has been shown for wheat and barley transcriptomes (Schreiber *et al.*, 2009), a limited number of genotype-specific genes would have potentially allowed the identification of constitutively expressed *R*-genes. However, the two genotypes showed very high numbers of differentially expressed genes (chapter 3) which may be mainly due to the obligate outbreeding fertilization of *L. multiflorum* (Spoor, 1976). Isogenic lines would have facilitated these analyses; however due to the unavailability of such lines, additional experimental exploration will be required in order to identify genomic regions or constitutively expressed *R*-genes that are up-regulated in the resistant genotype compared to the susceptible genotype. Mapping of these genes by means of high resolution melting curve analysis (HRM) on the *L. multiflorum* linkage map will allow for the identification of genomic regions that potentially contribute to bacterial wilt resistance.

6.2.2.2 Other approaches for candidate gene identification

Genomic and phenotypic approaches were used to understand the processes that determine the outcome of the interaction between *Xtg* and *L. multiflorum*. However, next to transcriptomics and marker-trait associations, there are additional approaches that may be used in order to identify major *R*-genes. These include, for example, comparative genomics. Due to close syntenic relationships among the members of the grass family (Devos, 2005), screening the *L. multiflorum* genome for homologous *R*-gene sequences that have been characterized in other

members of the grass family may represent a promising approach for *R*-gene identification. In general, *R*-genes are classified into five different classes (Baker *et al.*, 1997; Dangl & Jones, 2001; Hammond-Kosack & Jones, 1997). *Xanthomonas* resistance genes belonging to two of these five classes of genes have been found. The first class encodes cytoplasmic receptor-like proteins that contain a leucine-rich repeat (LRR) domain and a nucleotide-binding site (NBS). *Xanthomonas* resistance genes belonging to the NBS-LRR class are for example, *Bs4* (Schornack *et al.*, 2004), *Xa1* (Yoshimura *et al.*, 1998) and *Rxo1* (Zhao et al., 2004).

The other well characterized class of *Xanthomonas* resistance genes contain a transmembrane receptor with an extracellular LRR domain and an intracellular serine-threonine kinase, also called receptor-like kinases (RLK). The LRR domain serves as the receptor for specific extracellular molecules. RLK type proteins include the Xa21 protein (Song *et al.*, 1995) and the Xa26/Xa3 protein (Sun *et al.*, 2004). Due to close phylogenetic relationships of host and pathogen, the well characterized rice-*Xoo* system may represent an ideal model for the *L. multiflorum-Xtg* interaction. Comparative genomics and resistance gene analogue (RGA) screening has already enabled amplification of approximately 80 and mapping of seven RGA of Italian and perennial ryegrass (Ikeda, 2005; Li *et al.*, 2006a; Schejbel *et al.*, 2008).

In addition to comparative genomics, whole genome and RNA-sequencing (RNA-Seq; reviewed in Wang *et al.*, 2009) of the ryegrass genome and transcriptome using high-throughput DNA sequencing methods such as Roche 454 Life Sciences pyrosequencing, Illumina 1G or Applied Biosystems SOLiD (reviewed in Holt & Jones, 2008) may reveal promising *R*-gene candidates for bacterial wilt resistance. For RNA-Seq, the produced reads are aligned either to a reference genome or reference transcripts, or assembled *de novo* to produce a genome-scale transcription map that consists of transcriptional structure and/or expression level for each gene. Currently, whole genome sequencing of the *L. perenne* genome is being undertaken at Det Jorbrugvidenskabelige Fakultet (DJF), Aarhus University, Denmark. For future RNA-Seq studies in *L. multiflorum*, this genome may serve as an ideal reference genome. RNA-Seq has already been effectively used to analyze several plant transcriptomes such as *Arabidopsis thaliana*, rice, grape vine, glycine and cucumber (Ando & Grumet, 2010; Deyholos, 2010; Picardi *et al.*, 2010; Severin *et al.*, 2010; Zhang *et al.*, 2010) and further studies are to follow with the decreasing costs of current and future sequencing technologies. Although RNA-Seq is still in the early stages of use, it is expected to be advantageous for many applications and may completely replace microarrays for transcriptomics in the future.

6.2.3 Candidate genes of particular interest

Using different approaches for candidate gene identification in *L. multiflorum*, several genes of particular interest were discovered. For example, the low silicon transporter (Lsi1) and the germin-like protein 6 (GLP6) were revealed by transcriptome analyses of a resistant *L. multiflorum* genotype comparing *Xtg* infected with control-treated plants (chapter 3). In addition, candidate genes for susceptibility were identified comparing the transcriptomes of a susceptible and a resistant *L. multiflorum* genotype. Furthermore, comparative analyses of Xa21-like sequences from wheat and rice were used to identify a Xa21-like sequence in *L. multiflorum*. These identified candidate genes and their functions related to *Xtg* resistance first need to be validated, e.g. by means of overexpression or silencing in transgenic *L. multiflorum* genotypes. Once verified and screened for allelic variation, these candidate genes would represent valuable functional markers.

6.2.3.1 Silicon may increase resistance to biotic stress

A gene strongly up-regulated after *Xtg* infection is highly similar to a gene encoding the low silicon transporter Lsi1, a protein which belongs to a sub-family of aquaporins called Nodulin26-like major intrinsic protein. Lsi1 is known to be involved in silicon (Si) uptake in many plant species and Si is thought to be essential for resistance against biotic and abiotic stress (reviewed in Ma & Yamaji, 2006). Due to the fact that the exact role of silicon and its essentiality (or quasi-essentiality) for plants is largely unknown, this matter has recently attracted the interest of various researchers from different fields of plant science including plant nutrition, plant physiology and plant pathology. The more studies that are being conducted, the more beneficial effects of Si application are being reported concerning plant growth and amelioration of the ability to withstand all different kinds of plant stresses (biotic and abiotic).

Abiotic stress tolerances that are improved with Si application in either the soil or on leaves include drought, water logging, frost, salinity and heavy metal toxicity (reviewed in Liang *et al.*, 2007). Biotic stresses that Si provides some protection against, cover infections caused by various plant pathogens, but also herbivory attacks of arthropods and vertebrates (Fauteux *et al.*, 2006; Kvedaras *et al.*, 2009; Rodrigues *et al.*, 2004; Silva *et al.*, 2010). Si can improve host plant defense by enhancing natural enemy attraction through a change in volatile profile produced by plants when attacked by herbivores (Kvedaras *et al.*, 2009). However, it has been shown that Si is not beneficial to healthy plants and in order to provide an advantage, the plant needs to be under some form of imposed stress (Epstein, 2009; Fauteux *et al.*, 2006). In addition, Si has been shown to be able to act in a similar mode as

jasmonate and salicylate as a modulator of induced resistance that renders plant defense responses faster and more efficient (Brunings *et al.*, 2009; Fauteux *et al.*, 2006; Kvedaras *et al.*, 2009). Assuming that Lsi1 is responsible for Si uptake in forage grasses as it is in other plant species such as wheat and barley, the gene encoding Lsi1 would represent a promising candidate gene to overexpress in a transgenic *L. multiflorum* genotype which is highly susceptible to bacterial wilt. Increased bacterial wilt resistance in these transgenic genotypes would demonstrate the beneficial effect of increased Si transport in forage grasses and the potential of the *Lsi1* gene for MAS.

6.2.3.2 Germin-like proteins collocate with QTL for disease resistance

Another very promising candidate gene for bacterial wilt resistance identified by means of transcriptome analyses (chapter 3) is the germin-like protein (GLP) 6. Germins are a functionally diverse protein family with different enzyme activities (Woo *et al.*, 2000). All germins have the characteristic 'germin' motif involved in metal binding (Dunwell *et al.*, 2008). Germins belong to the large superfamily of cupins. Cupins are subgrouped into two different subclasses called monocupins and bicupins. Monocupins only contain one copy of the cupin domain and predominantly include plant oxalate oxidases and plant germins. On the other hand, bicupins have two copies of the cupin domain and include plant seed storage proteins and plant oxalate decarboxylases. and most germins share attributes such as resistance to extreme heat or denaturing agents; however, they differ in cell type specificity (Kim *et al.*, 2004). The differentially expressed GLP6 after *Xtg* infection belongs to the subclass of monocupins and according to homologies, it most likely belongs to the group of oxalate oxidase (OXO)-like GLPs (Carrillo *et al.*, 2009). In the past, several genes encoding OXOs and GLP have been associated with QTL in rice and barley (Faris *et al.*, 1999; Liu *et al.*, 2004; Ramalingam *et al.*, 2003; Wu *et al.*, 2004). However, the different functions of OXOs and GLP in basal defense responses have long been subject to various speculation. One of the predicted roles is that they facilitate cell wall strengthening through lignification and papillae formation. Another theory argues that the hydrogen peroxide (H_2O_2) produced after oxidation of oxalic acid (oxalate) is a reactive oxygen species that is directly toxic to pathogens (Wojtaszek, 1997). Finally, the third theory simply hypothesizes that this H_2O_2 is a signalling molecule that is involved in a number of disease response pathways. However, the literature is controversial about the contribution of OXOs and germins to the oxidative burst that occurs after elicitation of stress and pathogen recognition (Lane, 1994; Wojtaszek, 1997).

In rice, some evidence exists that differential gene expression of GLPs and OXOs among cultivars may be responsible for differential disease resistance (Carrillo *et al.*, 2009).

Transient silencing of the barley germin 4 (*HvGER4*) lead to increased susceptibility to powdery mildew but this effect has been demonstrated to be genotype dependent (Christensen *et al.*, 2004). Therefore, the level of partial resistance conferred by a particular genomic region varies across different genotypes and most likely depends on the allelic composition of other loci contributing to quantitative resistance. GLP6 silencing or overexpression in a number of *L. multiflorum* genotypes would show how and to which extent gene expression levels of GLP6 contribute to bacterial wilt resistance and whether the observed effect is genotype-dependent.

6.2.3.3 Xa21-homologous resistance mechanisms in *L. multiflorum*?

Another candidate gene of particular interest is the *Xa21*-homologous gene of *L. multiflorum*. Since *Xtg* resistance mechanisms in *L. multiflorum* have previously been hypothesized to be homologous to the *Xa21*-mediated resistance of rice effective against *Xoo* isolates (Song *et al.*, 1995; Studer *et al.*, 2006), *L. multiflorum* was screened for Xa21-homologous sequences. Using degenerate oligonucleotide primers on the basis of Xa21-like sequences of rice and wheat, a Xa21-like sequence was obtained (appendix). However, PCR positive *L. multiflorum* genotypes for the Xa21-like sequence were not significantly more resistant than PCR negative *L. multiflorum* genotypes. This indicated that this Xa21-like protein is not responsible for Xa21-like resistance mechanism in *L. multiflorum*.

6.2.3.4 Susceptibility genes rather than *R*-genes?

Numerous *Xanthomonas* resistance genes that do not belong to any of the five classes of *R*-genes (reviewed in Dangl & Jones, 2001) have been identified in rice. One example is the recessive *Xoo* resistance gene, *xa5*. *xa5* encodes the small subunit of the transcription factor IIA (TFIIAγ) with a mutation at position 39 in the second exon of the gene being responsible for resistance (Iyer & McCouch, 2004). Another example is the recessive *R*-gene, *xa13* which encodes a protein related to *MtN3*, encoding the nodulin 3 (N3) protein of *Medicago truncatula*. *MtN* genes have been shown to be up-regulated during the *Rhizobium*-induced nodulation process of *Medicago truncatula* (Gamas *et al.*, 1996). Due to the similarity to *MtN3* and its location on rice chromosome 8, the dominant allele Xa13 was named Os8N3. The difference between resistant (xa13/xa13) and susceptible plants is the elevated expression of Os8N3 during bacterial infection in susceptible plant genotypes and the absence of Os8N3 induction during bacterial infection in resistant genotypes (Yang *et al.*, 2006; Yuan *et al.*, 2009). Therefore, Os8N3 was concluded to be a susceptibility gene and bacterial strains that induce Os8N3 expression are virulent. Interestingly, three different genes similar to *MtN19* and *MtN21* were found to be up-regulated in the susceptible genotype compared to the

resistant genotype after *Xtg* infection and/or both after control-treatment and *Xtg* infection (electronic supplementary material of the Theoretical and Applied Genetics journal: http://www.springerlink.com/content/g1g253090347v0u2/122_2010_Article_1470_ESM.html). Similar to Os8N3, the genes in *L. multiflorum* encoding the nodulin proteins MtN19 and MtN21 could represent susceptibility genes of *L. multiflorum* expressed in susceptible genotypes. In the future, it may be a crucial aspect to consider when screening for *R*-genes that *MtN19* and *MtN21* expression is potentially related to susceptibility in *L. multiflorum* and recessive xa13-like resistance mechanisms could be present in resistant *L. multiflorum* genotypes. In order to test whether the non-induction of the *MtN19* and *MtN21* homologues in resistant *L. multiflorum* genotypes is responsible for *Xtg* resistance, qPCR analyses could be performed. *MtN19* and *MtN21* induction in susceptible *L. multiflorum* genotypes and the absence of induction in resistant genotypes after *Xtg* infection would provide strong evidence for susceptibility mechanisms. In addition to qPCR analyses, gene silencing of *MtN19* and *MtN21* in susceptible *L. multiflorum* genotypes could be performed in order to demonstrate whether silencing of *MtN19* and *MtN21* renders susceptible *L. multiflorum* genotypes more resistant.

6.3 *Xtg* – a *Xanthomonas* spp. with distinct features

Phytopathogenic bacteria belonging to the genus *Xanthomonas* have many general features and virulence associated characteristics in common. However, the significance of the virulence factors for the interaction with their hosts may differ greatly. Although *Xtg* shares some virulence associated characteristics with the other sequenced *Xanthomonas* spp. such as the ability to produce the extracellular polysaccharide (EPS) xanthan encoded on the *gum* gene cluster and a T3SS gene cluster, *Xtg* has been shown to be different from the other sequenced *Xanthomonas* spp. in many ways. On one hand, *Xtg* has broad host range when compared to the other sequenced *Xanthomonas* spp. which are mostly restricted to one or two plant species. In addition, a phylogenetic tree based on 16S ribosomal DNA sequences of *Xanthomonas* spp. (Hauben *et al.*, 1997) showed that *Xtg* is distantly related to the other sequenced *Xanthomonas* spp. Distinct phylogenetic relationships have also been demonstrated for the usually highly conserved genes encoding the T3SS components (chapter 5). The T3SS mediates the delivery of effector proteins which may suppress host defense mechanisms and promote virulence processes of the pathogen. Expression of the T3SS gene cluster is induced *in planta* by the HrpG/HrpX two-component regulatory system. In contrast to *hrpG* deficient mutants of other *Xanthomonas* spp. (Zou *et al.*, 2006; Cho *et al.*, 2008; Darsonval *et al.*,

2008), a *hrpG* deficient mutant of *Xtg* was not affected in terms of *in planta* survival and multiplication. On one hand, these distinct features of *Xtg* render this pathogen system especially complex to study; on the other hand, using other more suitable models than, for example, the other sequenced *Xanthomonas* spp. for comparisons may reveal further crucial insights into this host-pathogen system. Due to a very broad host range of *R. solanacearum* covering over 200 plant species and a relatively broad host range of *Xtg* (Egli *et al.*, 1975), and a similar *hrp* gene cluster (Salanoubat *et al.*, 2002; chapter 5), this host-pathogen system may represent a very suitable model for studying the *L. multiflorum*-*Xtg* interaction in the future. In addition, qualitative resistance against *R. solanacearum* in solanaceous crops has never been observed despite the identification of a major QTL on tomato chromosome 6 (Danesh *et al.*, 1994) indicating another similar feature compared to *Xtg* resistance in *L. multiflorum*.

6.3.1 The T3SS of *Xtg*

A *hrp* gene cluster encoding the T3SS apparatus components was identified in the genome of *Xtg*29 (chapter 5). The length and orientation of these genes is similar to the T3SS of other sequenced *Xanthomonas* spp. (Fig. 5.2) with the exception that the two genes encoding the two-component regulatory system *hrpG* and *hrpX* are located within the T3SS gene cluster (chapter 5). In the genomes of the other sequenced *Xanthomonas* spp. with a T3SS, the two-component regulatory system for *hrp* gene regulation *hrpG*/ *hrpX* are located together outside of the *hrp* gene cluster. Similar to *Xtg* in the β-proteobacterial plant pathogen *Ralstonia solanacearum* isolate GMI1000, the *hrpG* and *hrpX* genes are also located within the T3SS gene cluster (Salanoubat *et al.*, 2002). Preliminary quantitative real-time PCR analyses with plant material of the *hrpE* and *hrcR* genes of *Xtg*29 and the *ΔhrpG* mutant *Xtg* indicated that the T3SS components are expressed and are functional in *Xtg*.

6.3.2 The role of the *hrpG* gene for pathogenicity and host colonization

Preliminary analyses using quantitative PCR (qPCR) targeting the *hrpE* gene which encodes the pilus protein of the T3SS and *hrcR* gene encoding a basal structural protein of the T3SS (chapter 5) in plants infected with *ΔhrpG* mutant and the wildtype strain showed significantly reduced expression levels of the *hrpE* gene of *Xtg* in plants infected with *ΔhrpG* mutant. *hrcR* expression levels of *Xtg* were also lower but not significant in plants infected with the *ΔhrpG* mutant. This indicated that similar to other *Xanthomonas* spp., the *hrpG* gene is responsible for activation of the expression of T3SS genes in *Xtg*. However, alternative signalling for the control of gene expression of the *hrpC* and *hrpE* operons has been demonstrated for one copy

of a ColR/ColS two-component regulatory system of *Xanthomonas campestris* pv. *campestris* (Zhang *et al.*, 2008). One ColR/ColS two-component regulatory system has also been identified in the *Xtg*29 genome (chapter 5) which could also function as alternative signaling system for *hrp* gene expression. Whether a ColR/ColS two-component regulatory system can substitute the HrpG/HrpX two-component regulatory system could be investigated by means of qPCR analyses of *colR* and *colS* different time points after *Xtg* infection and including further genes encoding T3SS structural proteins.

Beside T3SS activation, the HrpG/HrpX two-component regulatory system also has been shown to play a crucial role for other factors potentially associated with pathogenicity and host adaptation. These include type II secretion, the control of expression of lectins, and the biosynthesis of polyamines and phytohormones, such as ethylene and auxin (Wang *et al.*, 2008). Using proteomic analyses, it has been demonstrated that *hrpG* regulates at least 11 genes associated with the T2SS in *Xanthomonas axonopodis* pv. *citri* (Yamazaki *et al.*, 2008). Therefore, reduced symptoms of plants infected with the *ΔhrpG* mutant could also be hypothesized to be connected with deficiency of T2SS regulation and virulence factor secretion. However, in order to clarify the involvement of the *hrpG* gene and the T3SS in general for bacterial wilt infection requires additional experimental data and will provide necessary insights into bacterial virulence factors, symptom development and *in planta* survival of the *ΔhrpG* mutant.

6.3.3 *avr* genes and TALEs as potential plant resistance gene counterparts

A number of identified effectors in *Xtg*29 are similar to avirulence (*avr*) genes of other *Xanthomonas* spp. and induce a hypersensitive response (HR) in certain hosts. These include, for example AvrBs2 or AvrRxv for which the *R*-gene counterparts have been identified in other plant species. For example, the *R*-gene *Bs2* (Minsavage *et al.*, 1990) has been identified in pepper and a *AvrRxv* interactor 1 (ARI1) protein (Whalen *et al.*, 2008) has been identified in tomato lines being the counterparts to *Xcv* Avr determinants AvrBs2 and AvrRxv. Interestingly, the presence and evolution of *avr* genes is often associated with bacteria which are limited to a specific host range (Leach & White, 1996). However, *Xtg* has a broad host range and is virulent on the forage grasses belonging to *Lolium* spp., *Phleum* spp. and *Poa* spp. but not on *Arrhenaterum* spp. (Egli & Schmidt, 1982). Nevertheless, *avr* genes were identified in *Xtg*29 which may imply that these Avr proteins confer some selective advantages on a diverse set of host plants (Kjemtrup *et al.*, 2000). Also in the *Ralstonia solanacearum* genome, 14 CDS were identified to encode Avr determinants despite the absence of qualitative resistance in solanaceous crops to bacterial wilt caused by *R. solanacearum*

(Salanoubat *et al.*, 2002). Due to the similarities including the organization the T3SS gene cluster and effector and Avr determinant similarity, this analysis of *R. solanacearum* may be of major interest for understanding the interaction of *Xtg* with *L. multiflorum*.

A number of Avr proteins of other *Xanthomonas* spp. belong to the transcription activator-like effectors (TALE) or *avrBs3/pthA* family. In this context, plant *R*-genes either specifically recognize TALE structure (e.g. rice *Xa1* or tomato *Bs4*), subvert TALE function (e.g. rice *Xa27* and pepper *Bs3*) or interfere with TALE activity (e.g. rice *xa5* and rice *xa13*; reviewed in Bogdanove *et al.*, 2010). TALEs are highly conserved and usually solely differ in the number of near-identical, 34-amino-acid, direct repeats in the central portion of the protein known as the repetitive region (Fig. 6.1; Bogdanove *et al.*, 2010). The repetitive region defines specificity and polymorphisms within the repeat region occur primarily at positions 12 and 13. This region is also called the repeat-variable diresidue (RVD). It has been shown that one RVD (2 amino acids) of a TALE corresponds directly to one nucleotide in their target sites (the promoter) of the corresponding *R*-genes (Fig. 6.1). Therefore, depending on the amount of repeats and TALE specificity, this may enable direct target site prediction within plant genomes and therewith direct *R*-gene or susceptibility gene identification (Moscou & Bogdanove, 2009).

Judging from these analyses of *Xanthomonas* TALE RVD and *R*-gene promoter sequence, the existence and detection of TALEs on the *Xtg*29 genome is of major interest for the identification of possible target sites of *R*-genes in *L. multiflorum*. So far, no coding sequences or effectors with sequence similarity to TALEs have been found in *Xtg*29 by whole genome analyses (chapter 5). Nevertheless, it cannot be excluded that TALEs in *Xtg*29 exist due to numerous gaps between contigs of the draft genome sequence. Therefore, it will be of major importance to sequence gaps (especially greater gaps that could harbour genes encoding TALEs) and identify further effectors. Together with the analysis presented in chapter 5, finding further *avr* genes and TALEs in *Xtg* could enable the elucidation of resistance mechanisms in *L. multiflorum* and could be of major interest in the future.

Figure 6.1 ***top:*** The transcription activator-like effector (TALE)–DNA recognition cipher. A generic TALE showing the amino acid repeat region (open boxes) and a representative repeat sequence with the RVD underlined. ***bottom:*** Best pattern matches (low-entropy alignments) for several TALE repeat-variable diresidue (RVD) and target gene promoter sequences. The asterisk indicates a deletion at residue 13 (Moscou & Bogdanove, 2009).

6.3.4 Other effector proteins of *Xtg*

Comparative analyses with genes encoding effector proteins of other sequenced *Xanthomonas* spp. revealed 22 genes for putative T3SS effectors. The identified genes were for the most part homologous to *Xanthomonas* outer proteins (*xop*) or *hrp*-dependent outer proteins (*hop*) of *Pseudomonas* spp. In addition, there are some effector proteins that have not been described in detail and revealed similarity to putative effectors of *Ralstonia solanacearum*. Despite the identification of a diverse pool of genes encoding effector proteins, it is expected that even more genes encoding effector proteins are present in *Xtg* which are not homologous to *Xanthomonas* or *Pseudomonas* effectors. There are various tools that have been developed

for screening entire sets of protein sequences derived from predicted coding sequences (CDS) of genomes for T3SS effector proteins (Arnold *et al.*, 2009; Burstein *et al.*, 2009; Löwer & Schneider, 2009; Samudrala *et al.*, 2009). These tools use machine learning techniques to train on sets of known secreted proteins. Predicted protein sequences are screened for N-terminal domains that are important for secretion through the T3SS. Residues 1–25 are thought to contain a region with a secretion signal. In addition, some effectors contain a chaperone binding domain that spans residues 25–100 (Ghosh, 2004). The methods predict secreted effectors with high specificity and sensitivity (McDermott *et al.*, 2011). Using such tools may support the identification of additional T3SS effector proteins and may enable the identification of novel effectors. In addition to secretion signals, many genes that are regulated in a *hrp*-dependent manner by the two-component regulatory system HrpG/HrpX harbour a PIP (plant-inducible promoter) box with a consensus motif (TTCGB-[N15]-TTCGB-[N30-32]-YANNNT) in their promoter sequence (Fenselau & Bonas, 1995). Although several *xop* genes that are controlled by HrpG and HrpX do not contain PIP boxes (reviewed in Büttner, 2002), screening the *Xtg* genome for such PIP box motifs may represent another promising approach to identify additional *hrp*-dependently regulated genes and virulence factors that are induced when *Xtg* are inside the host plants.

6.4 Conclusions

The genomic and transcriptomic approaches presented in this thesis have revealed novel insights into bacterial wilt resistance of *L. multiflorum* and *Xtg* virulence. Taken together, the results of the race-specificity experiment, the marker-trait associations, and the transcriptome analyses indicated that in distantly related germplasm *Xtg* resistance is conferred by various resistance mechanisms and genes that contribute to resistance quantitatively. Two independent analyses revealed two different genomic regions (i.e. one on LG 4 (Studer *et al.*, 2006) and one on LG 5 described in chapter 2) to contribute to a significant degree to *Xtg* resistance and in addition, the transcriptome analyses revealed two interesting candidate genes for *Xtg* resistance such as *Lsi1* and *GLP6*. To shed light on the *L. multiflorum*-*Xtg* interaction from a different perspective, virulence factors of *Xtg* were investigated. Interactions between *R. solanacearum* and solanaceous crops may serve as more suitable models due to comparative analyses that revealed a T3SS with a genetic organization different from other sequenced *Xanthomonas* spp., but very similar to the β-proteobacterial plant pathogen *Ralstonia solanacearum*. In contrast to other *hrpG* deficient *Xanthomonas* strains of other species, the *hrpG* gene of *Xtg*29 is not crucial for *in planta* multiplication and survival,

highlighting the distinctness of the *Xtg-L. multiflorum* interaction. The analyses of *Xtg* presented in this thesis provide a first very informative insight into possible virulence mechanisms. Combined, the approaches of *L. multiflorum* resistance mechanisms and *Xtg* virulence factors have allowed to gain a more profound understanding of this host-pathogen system and will enable the identification of further candidate genes and the development of MAS tools in the future.

6.5 Outlook

Approaches to further identify genomic regions that contribute to *Xtg* resistance in *L. multiflorum* will include mapping of promising genes identified by transcriptome analyses on the *L. multiflorum* linkage map (Studer *et al.*, 2006), for example by means of high resolution melting curve (HRM) or SNP analyses with the mapping population derived from a pseudo-testcross family. To identify isolate-specific virulence mechanisms of *Xtg*, further steps could include detailed analyses of the T3SS and effector translocation. Additional qPCR analyses and mutants of genes encoding T3SS components could provide valuable information concerning T3SS regulation and functionality. A PCR screen of other *Xtg* isolates for the same genes encoding T3SS effectors and/or avirulence genes identified in the genome of *Xtg*29 (chapter 5) could be developed to identify virulence factors that render the different *Xtg* isolates more or less virulent. In addition, whole genome sequencing and comparative analyses of additional *Xtg* isolates with the draft genome sequence of *Xtg*29 will enable the identification of additional virulence factors. Moreover, whole genome sequencing of an isolate of a pathovar with specific virulence to the forage grass *Arrhenatherum elatius* (i.e. *Xanthomonas translucens* pv. *arrhenatheri* (*Xta*)) may additionally enable the identification of virulence factors that define and affect host-specificity. Together with the work presented in this thesis, further data exploration will add significant value to the understanding of this host-pathogen system. Most evident steps for understanding in more detail the *L. multiflorum*-*Xtg* interaction include:

- testing of *Xtg* mutants deficient of genes encoding different T3SS structural components by means of a pathogenicity screen in the greenhouse on *L. multiflorum* plants.
- performing prediction of T3SS effector proteins using a T3SS effector prediction tool (proposed in 6.5.3) such as the SVM-based identification and evaluation of virulence effector (SIEVE) tool (Samudrala *et al.*, 2009).
- searching the *Xtg*29 genome for TALEs and additional avirulence genes for targeted *R*-gene identification in *L. multiflorum* by means of proteins sequence comparison with TALEs of other *Xanthomonas* spp.
- overexpression and/or gene silencing of candidate genes in transgenic *L. multiflorum* genotypes.

Appendix: Expression of a Xa21-like protein in *Lolium multiflorum*

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1. Introduction

Xanthomonas translucens pv. *graminis* (*Xtg*) is the causal agent of bacterial wilt on forage grasses (Egli *et al.*, 1975). The disease is responsible for serious annual yield and quality losses in forage production (Suter *et al.*, 2005). Breeding for resistance is the only commonly applied means of disease control, but further advances through phenotypic selection are limited, and susceptible individuals still occur in advanced breeding material. The identification of resistance genes (*R*-genes) in *L. multiflorum* therefore represents a major objective and may provide the basis for the implementation of marker assisted selection (MAS) in *L. multiflorum*. In rice, MAS has been widely implemented in resistance breeding against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and is frequently applied to select for the presence of specific *R*-genes that confer resistance to different isolates (Perumalsamy *et al.*, 2010).

A number of these *R*-genes belong to the large class of resistance genes encoding a nucleotide binding site (NBS) plus a leucine-rich repeat (LRR) domain (e.g. *Xa1*; Yoshimura *et al.*, 1998). NBS-LRRs are presumably cytoplasmatic and the NBS part is a critical element for the catalytic activity of proteins and is required for ATP- and GTP- binding (Saraste *et al.*, 1990). In general, NBS-LRRs block bacterial pathogenesis by recognition of Avr proteins secreted through the type III secretion system (T3SS) of *Xanthomonas* spp. Another class of resistance proteins of rice, receptor-like kinases (RLK), carry a leucine rich repeat (LRR) in the putative extracellular domain, a single pass transmembrane domain, and a serine/threonine kinase intracellular domain. A major *R*-gene of this class in rice encodes Xa21. Xa21 confers race-specific resistance to all *Xoo* isolates secreting the AvrXa21 protein (reviewed in Niño-Liu *et al.*, 2006; see 6.3.4). Since many *Xoo* isolates secrete the AvrXa21 protein, Xa21-mediated resistance is effective against a broad range of different *Xoo* strains (Wang *et al.*, 1996). Xa21-mediated *Xoo* resistance increases from the juvenile 2-leaf stage to the adult stage and is therefore developmentally controlled. However, Xa21 expression has been shown to be neither correlated with development, nor has it been induced upon infection with *Xoo* or wounding (Century *et al.*, 1999). Therefore, there is strong evidence that it is constitutively expressed in all plants carrying the *Xa21* gene. Transgenic lines expressing the *Xa21* gene have been shown to be resistant to the same *Xoo* isolates as the donor cultivar indicating same gene-for-gene specificity (Wang *et al.*, 1996). Beside Xa21, only one additional plant receptor-like kinase (RLK) protein (i.e. Xa3/Xa26) has been characterized in rice (Sun *et al.*, 2004). Other RLK resistance proteins include Pto of tomato which provides resistance to *Pseudomonas syringae* (Salmeron *et al.*, 1996).

In *L. multiflorum*, quantitative trait loci (QTL) analysis of a pseudo-testcross family has indicated that resistance to *Xtg* may be controlled by one major QTL on linkage group (LG) 4 which has explained up to 84% of the total phenotypic variance for *Xtg* resistance (Studer *et al.*, 2006). In addition to this major QTL, three minor QTL on LG 1, 5 and 6 were identified, explaining between 2.9 and 7.4% of the total phenotypic variance. In a number of plant species, the existence of major QTL has been associated with the presence of one or only few major resistance genes (Mutlu *et al.*, 2005; Verdier *et al.*, 2004; Yang & Francis, 2005). LG 4 has been shown to be syntenic to the rice chromosomes 11 and 3 (Devos, 2005). In rice, the resistance gene Xa21 has been shown to be localized on chromosome 11 (Song *et al.*, 1995). Due to syntenic relationships between the members of the grass family (*Poaceae*), these the rice gene encoding Xa21 may represent an ideal model to study receptor-like kinase homologues in *L. multiflorum*.

Since the major QTL on LG 4 indicated the existence of Xa21-like resistance mechanisms, the objective of the present study was to search the *L. multiflorum* genome for Xa21-like sequences. Primers were designed on a consensus sequence of a gene encoding the Xa21-like protein of *Triticum aestivum* and the *Xa21* gene of *Oryza sativa* and the presence of this sequence was amplified from a number of *L. multiflorum* genotypes previously screened for *Xtg* resistance (see chapter 2).

2. Materials and Methods

The plants of the 59 used *L. multiflorum* genotypes, the 6 used *Xtg* isolates and the area under the disease progress curve value (AUDPC) calculations are described in chapter 2. The extraction of genomic DNA was performed as described in chapter 2. PCR reactions were conducted using Hotstar and GoTaq DNA Polymerase (Qiagen; Hilden, Germany and Promega, Madison, WI, USA) as described in chapter 4 and chapter 2. The *L. multiflorum* genotype *Lm*B-01 which previously has been shown to be highly resistant to *Xtg* was used for the primary screen of the Xa21_forward and Xa21_reverse primer. The sequences of both the Xa21-like protein from *Triticum aestivum* (GenBank accession: EU423904) and the gene encoding the Xa21 resistance protein of *Oryza sativa* (GenBank accession: AB212798) were retrieved from GenBank (http://ncbi.nlm.nih.gov/) and aligned using the CLUSTALW function in Bioedit (version 7; Hall, 1999). Primers were designed based on the consensus sequence of the partial alignment of EU423904 and AB212798 (Fig. 2). The PCR product for sequencing was extracted from the agarose gel (1%) using the Qiaquick gel extraction kit (Qiagen) the product was sequenced on a ABI3130 (Applied Biosystems) using BigDye v.1.1 (Applied Biosystems) as described in 4.3.3.

3. Results and Discussion

The resulting primers Xa21_forward and Xa21_reverse (Fig. 2) of the pairwise alignment of the gene encoding an Xa21-like protein from *Triticum aestivum* (EU423904) and a part of the gene encoding the Xa21 resistance protein of *Oryza sativa* (AB212798; i.e. Xa21_forward: AWGTKGCAGTGAAGGTWYT and Xa21_reverse: KRTGWARATAGTCCAGTGC) were tested on genomic DNA of the *L. multiflorum* genotype *Lm*B-01 which previously has been shown to be highly resistant to *Xtg* in a screening experiment with different *Xtg* isolates (see chapter 2). Multiple sequence alignment (CLUSTALW) of the gene encoding an Xa21-like protein from *Triticum aestivum* (EU423904) and a part of the gene encoding the Xa21 resistance protein of *Oryza sativa* (AB212798) showed an average nucleotide identity of 51.9%. PCR amplification using the primers Xa21_forward and Xa21_reverse revealed among other products, a product of the expected length of approximately 300 bp (Fig. 1).

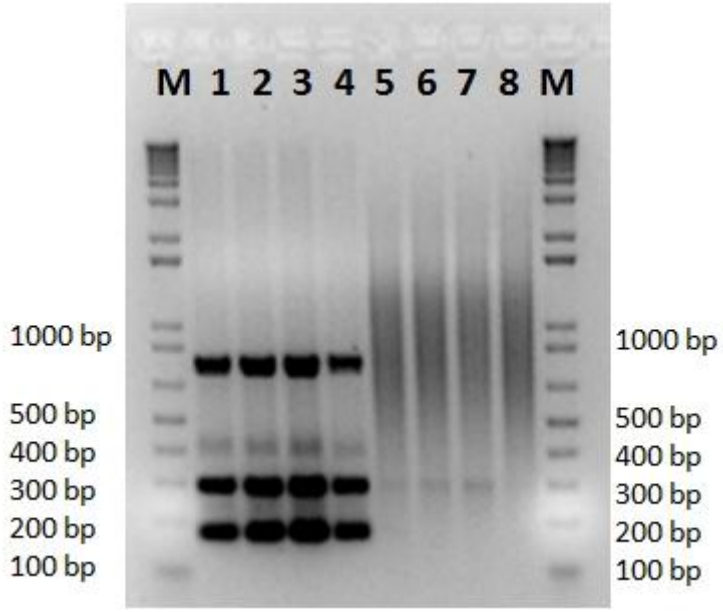

Figure 1 PCR amplification with the Xa21_forward and Xa21_reverse primers. M: 1kb plus DNA Ladder (Invitrogen). Lanes 1 – 4: PCR reactions performed on genomic DNA of *L. multiflorum* genotype *Lm*B-01. Lanes 5 – 8: PCR reactions performed on cDNA of *L. multiflorum* genotype *Lm*B-01.

Sequencing of the amplified PCR product with the same primers revealed a sequence similar to the Xa21-like protein sequence of *L. multiflorum.* The sequence identity matrix of the CLUSTALW alignment revealed a sequence identity of the *L. multiflorum* Xa21-like sequence of 80.0% with the *T. aestivum* sequence and of 56.0% with the *O. sativa* sequence. Thus, comparative sequence analyses revealed that the sequence of *L. multiflorum* was more similar to *T. aestivum* than to *O. sativa* (Fig 2*).* The primers that are specific for *L.*

multiflorum i.e. LmXa21_forward and LmXa21_reverse, were designed after sequencing with the Xa21_forward and Xa21_reverse primers.

Based on the sequenced fragment, a new primer pair specific for *L. multiflorum* (Fig. 2; i.e. LmXa21_forward: TTTTAACCTTGAGATGCCAGG and LmXa21_reverse: TTGTGGTGAAGCCACGTGT) amplifying a product of approximately 200 bp was designed. PCR reactions on genomic DNA of 59 *L. multiflorum* genotypes representing commercially available cultivars, cultivar candidates from Agroscope Reckenholz-Tänikon, F_2 progeny of a mapping population (Studer *et al.*, 2006) and ecotypes sampled in Switzerland and previously used for elucidating race-specific interactions between *Xtg* isolates and *L. multiflorum* genotypes, and gel electrophoresis indicated the presence or absence of this sequence (Table 1). In order to test whether the sequence is also expressed in *L. multiflorum* and not only present on genomic DNA, the primers were also tested on cDNA samples prepared from RNA extracts of genotypes *Lm*B-01 and *Lm*K-03 as described in chapter 3.

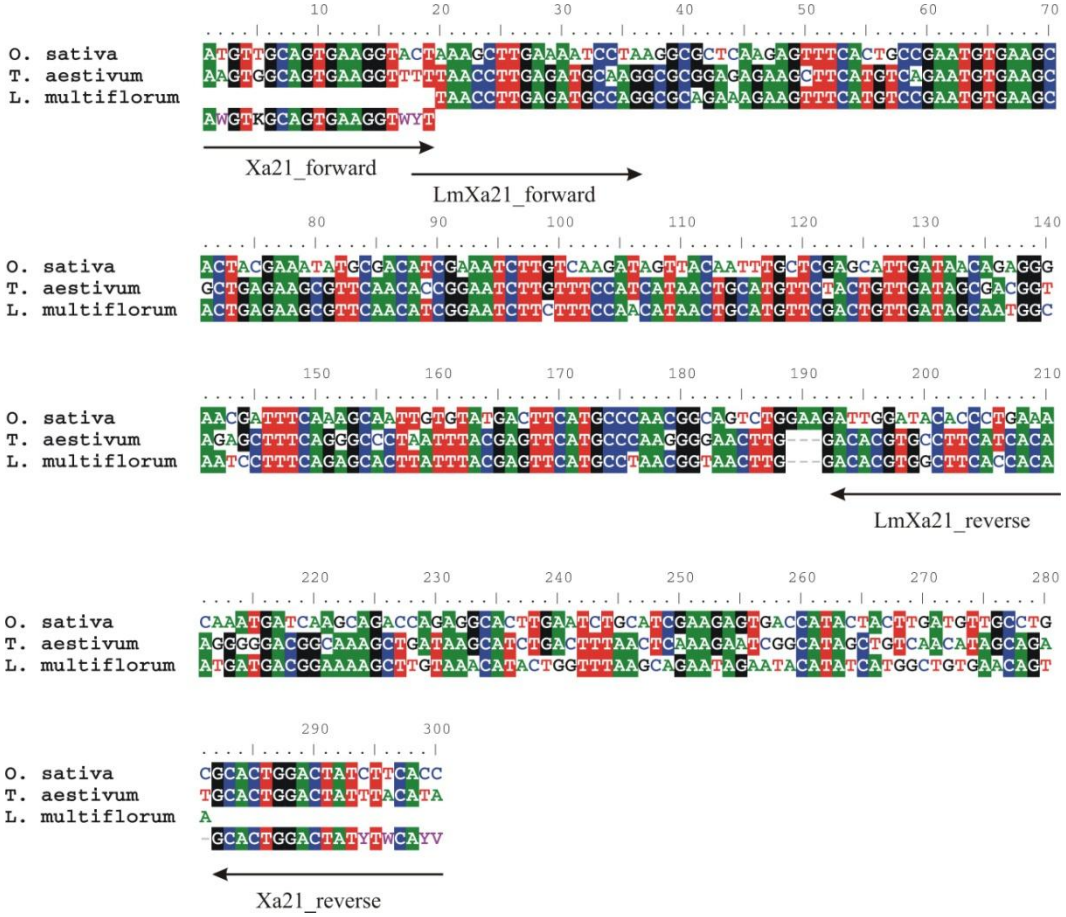

Figure 2 Multiple sequence alignment (CLUSTALW) of a gene encoding a Xa21-like protein of *Triticum aestivum* (GenBank accession: EU423904) and the gene encoding the Xa21 resistance protein of *Oryza sativa* (GenBank accession: AB212798) obtained from GenBank. The sequenced part of a gene encoding a Xa21-like protein of *Lolium multiflorum* was added to the alignment using the CLUSTALW function in Bioedit (version 7; Hall, 1999). Starting positions and length of designed primers are indicated with arrows.

Resistance of the 59 *L. multiflorum* genotypes was ranked according to the mean AUDPC values resulting from infection with six different *Xtg* isolates (chapter 2). In addition, the plants were classified according to the criteria described in chapter 2 into resistant (R), moderately susceptible (MS) and susceptible (S). Successful amplification was obtained for 20 of the 59 genotypes (33.90%; Table 1). The number of genotypes where the Xa21-like protein sequence was present differed among the different populations. For example, the Xa21-like protein sequence could not be detected for any of the genotypes of population A, which consisted of genotypes of F_2 progeny of a mapping population segregating for bacterial wilt resistance (Studer *et al.*, 2006), and population K, composed of individuals from the commercially available highly susceptible cultivar Adret (Table 1). When comparing the

genotypes of population J which consisted of individuals from the commercially available cultivar Turilo, the most resistant genotype *Lm*J-03 revealed a Xa21-like sequence, whereas four other genotypes did not and are more susceptible (Table 1)

Table 1 PCR positive (+) and PCR negative (-) *Lolium multiflorum* genotypes with primers LmXa21_forward and Xa21_reverse and AUDPC values caused by *Xanthomonas translucens* pv. *graminis* infection. The genotypes are sorted according to the AUDPC values with the most resistant genotype being on the top. Classification symbols indicate R: resistant, MS: moderately susceptible, and S: susceptible.

L. multiflorum **genotype name**	***L. multiflorum*** **population**	**PCR with LmXa21 primers**	**Mean AUDPC value**	**Classification**
LmL-03	L	-	31.06	R
LmB-01	B	+	31.43	R
LmI-03	I	-	32.17	R
LmB-05	B	+	33.25	R
LmA-01	A	-	33.60	R
LmI-01	I	-	33.70	R
LmA-10	A	-	33.90	R
LmJ-03	J	+	34.31	R
LmA-03	A	-	35.65	R
LmA-05	A	-	35.71	R
LmG-02	G	-	35.83	R
LmH-03	H	+	36.33	R
LmC-01	C	-	36.35	R
LmI-02	I	-	36.39	R
LmC-04	C	-	36.67	R
LmC-02	C	-	36.90	R
LmF-04	F	+	36.94	R
LmL-04	L	-	37.13	R
LmB-03	B	-	37.20	R
LmL-05	L	-	37.31	R
LmA-12	A	-	37.36	R
LmB-02	B	-	37.44	R
LmH-02	H	+	37.81	R
LmA-04	A	-	38.14	R
LmF-02	F	+	38.79	R
LmA-11	A	-	38.85	R
LmJ-02	J	-	38.88	R
LmH-04	H	+	39.13	MS
LmD-03	D	-	39.48	MS
LmB-04	B	+	39.52	MS
LmA-07	A	-	39.85	MS

L. multiflorum **genotype name**	***L. multiflorum*** **population**	**PCR with LmXa21 primers**	**Mean AUDPC value**	**Classification**
LmE-04	E	-	40.25	MS
LmH-01	H	-	40.27	MS
LmC-03	C	+	40.90	MS
LmG-01	G	+	41.04	MS
LmC-05	C	+	41.88	MS
LmK-05	K	-	41.94	MS
LmD-04	D	-	41.98	MS
LmD-02	D	+	42.00	MS
LmD-01	D	-	42.21	MS
LmE-03	E	+	42.79	MS
LmL-01	L	+	42.84	MS
LmG-03	G	+	43.21	MS
LmJ-05	J	+	43.83	MS
LmE-02	E	-	44.40	MS
LmF-01	F	+	46.21	MS
LmK-04	K	-	46.50	MS
LmA-08	A	-	47.13	MS
LmA-02	A	-	47.29	MS
LmA-09	A	-	48.96	MS
LmF-03	F	-	49.11	MS
LmI-04	I	+	52.00	MS
LmE-01	E	+	53.25	MS
LmJ-01	J	-	58.91	MS
LmK-06	K	-	60.10	S
LmJ-04	J	-	70.10	S
LmK-02	K	-	77.31	S
LmK-03	K	-	82.67	S
LmK-01	K	-	83.72	S

Based on the AUDPC values, and the positive or negative LmXa21-PCR,. a two-sided t-test was performed in order to test if AUDPC values of plants with the Xa21-like protein differed significantly from AUDPC values of plants lacking the sequence for the Xa21-like protein. This t-test revealed no significant difference between AUDPC values of plants with the Xa21-like protein and plants without the sequence for the Xa21-like protein (P=0.189). Xa21-PCR on cDNA prepared from the resistant *L. multiflorum* genotype *Lm*B-01 (Fig. 1) was positive whereas the susceptible genotype *Lm*K-01 was negative (data not shown). The PCR fragment had a length of 300 bp (the same fragment as is obtained when amplifying from genomic DNA; Fig. 1), demonstrating that this sequence is also expressed in *L. multiflorum* and indicating that there are no introns in this region.

From the genomic DNA of genotypes derived from the F_2 progeny of a mapping population segregating for bacterial wilt resistance (Studer *et al.*, 2006), no sequence encoding a Xa21-like protein was amplified. The mapping population of Studer *et al,* (2006) consists of F_1 progeny of one genotype from the highly susceptible cultivar Adret, and the

other genotype was selected from advanced breeding germplasm (M2289) with a high level of bacterial wilt resistance. In this mapping population, one major QTL indicating major *R*-gene mediated *Xtg* resistance was detected. Since a sequence encoding a Xa21-like protein was not found in the F_2 progeny, it is concluded that either only twelve F_2 progeny genotypes were selected that inherited the allele for susceptibility from the Adret grandparent or this Xa21-like protein is not responsible for the major QTL in this mapping population or by coincidence.

4. Conclusions

It was demonstrated that a putative gene encoding a Xa21-like protein was present in 20 out of 59 *L. multiflorum* genotypes. The gene is highly similar to the gene encoding a Xa-21-like protein of *T. aestivum* and moderately similar to the *Xa21* gene of *O. sativa*. Expression of this sequence is not primarily responsible for major *R*-gene mediated resistance in *L. multiflorum*; however, it may contribute to *Xtg* resistance. However, since the presence of a coding region on the genome does not necessarily imply expression of this sequence, it could be highly interesting to use the developed tool to quantify Xa21-like gene expression by real-time PCR on cDNA of all the 59 different *L. multiflorum* genotypes and correlate expression levels to *Xtg* resistance.

7 References

Abramovitch, R. B., Anderson, J. C. & Martin, G. B. (2006). Bacterial elicitation and evasion of plant innate immunity. *Nature Reviews Molecular Cell Biology* **7**, 601-611.

Agrios, G. N. (2005). *Plant Pathology*, Fifth edn: Elsevier Academic Press.

Aharoni, A. & Vorst, O. (2002). DNA microarrays for functional plant genomics. *Plant Molecular Biology* **48**, 99-118.

Aldaoud, R., Anderson, M. W., Reed, K. F. M. & Smith, K. F. (2004). Evidence of pathotypes among Australian isolates of crown rust infecting perennial ryegrass. *Plant Breed* **123**, 395-397.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic Local Alignment Search Tool. *Journal Of Molecular Biology* **215**, 403-410.

An, Q. L., Ehlers, K., Kogel, K. H., van Bel, A. J. E. & Huckelhoven, R. (2006). Multivesicular compartments proliferate in susceptible and resistant MLA12-barley leaves in response to infection by the biotrophic powdery mildew fungus. *New Phytologist* **172**, 563-576.

Andersen, J., Jensen, L., Asp, T. & Lübberstedt, T. (2006). Vernalization response in perennial ryegrass (*Lolium perenne* L.) involves orthologues of diploid wheat (*Triticum monococcum*) *VRN1* and Rice (*Oryza sativa*) *Hd1*. *Plant Molecular Biology* **60**, 481.

Andersen, J. R. & Lübberstedt, T. (2003). Functional Markers in Plants. *TRENDS in Plant Science* **8**, 554-560.

Ando, K. & Grumet, R. (2010). Transcriptional profiling of rapidly growing cucumber fruit by 454-pyrosequencing analysis. *Journal of the American Society for Horticultural Science* **135**, 291-302.

Armstead, I. P., Turner, L. B., Farrell, M., Skot, L., Gomez, P., Montoya, T., Donnison, I. S., King, I. P. & Humphreys, M. O. (2004). Synteny between a major heading-date QTL

in perennial ryegrass (*Lolium Perenne* L.) and the HD3 heading-date locus in rice. *Theoretical and Applied Genetics* **108**, 822-828.

Arnold, D. L., Pitman, A. & Jackson, R. W. (2003). Pathogenicity and other genomic islands in plant pathogenic bacteria. *Mol Plant Pathol* **4**, 407-420.

Arnold, R., Brandmaier, S., Kleine, F., Tischler, P., Heinz, E., Behrens, S., Niinikoski, A., Mewes, H. W., Horn, M. & Rattei, T. (2009). Sequence-based prediction of type III secreted proteins. *PLoS Pathogens* **5**.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M. & Sherlock, G. (2000). Gene Ontology: tool for the unification of biology. *Nature Genetics* **25**, 25-29.

Asins, M. J. (2002). Present and future of quantitative trait locus analysis in plant breeding. *Plant Breed* **121**, 281-291.

Aslam, S. N., Newman, M. A., Erbs, G., Morrissey, K. L., Chinchilla, D., Boller, T., Jensen, T. T., De Castro, C., Ierano, T., Molinaro, A., Jackson, R. W., Knight, M. R. & Cooper, R. M. (2008). Bacterial polysaccharides suppress induced innate immunity by calcium chelation. *Current Biology* **18**, 1078-1083.

Asp, T., Frei, U. K., Didion, T., Nielsen, K. K. & Lübberstedt, T. (2007). Frequency, type, and distribution of EST-SSRs from three genotypes of *Lolium perenne*, and their conservation across orthologous sequences of *Festuca arundinacea*, *Brachypodium distachyon*, and *Oryza sativa*. *BMC Plant Biology* **7**.

Astua-Monge, G., Minsavage, G. V., Stall, R. E., Davis, M. J., Bonas, U. & Jones, J. B. (2000). Resistance of tomato and pepper to T3 strains of *Xanthomonas campestris* pv. *vesicatoria* is specified by a plant-inducible avirulence gene. *Molecular Plant-Microbe Interactions* **13**, 911-921.

Ausubel, F., Brent, R., Kinston, R., Moore, D., Seidman, J., Smith, J. & Struhl, K. (1987). *Current protocols in molecular biology*. New York: J. Wiley & Sons.

Bachem, C. W. B., Vanderhoeven, R. S., Debruijn, S. M., Vreugdenhil, D., Zabeau, M. & Visser, R. G. F. (1996). Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *Plant Journal* **9**, 745-753.

Bachem, C. W. B., Horvath, B., Trindade, L., Claassens, M., Davelaar, E., Jordi, W. & Visser, R. G. F. (2001). A potato tuber-expressed mRNA with homology to steroid dehydrogenases affects gibberellin levels and plant development. *Plant Journal* **25**, 595-604.

Baker, B., Zambryski, P., Staskawicz, B. & Dineshkumar, S. P. (1997). Signaling in Plant-Microbe Interactions. *Science* **276**, 726-733.

Balaji, V., Gibly, A., Debbie, P. & Sessa, G. (2007). Transcriptional analysis of the tomato resistance response triggered by recognition of the *Xanthomonas* type III effector *AvrXv3*. *Funct Integr Genomics* **7**, 305-316.

Bar-Or, C., Czosnek, H. & Koltai, H. (2007). Cross-species microarray hybridizations: a developing tool for studying species diversity. *Trends in Genetics* **23**, 200-207.

Becher, M., Talke, I. N., Krall, L. & Kramer, U. (2004). Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant Journal* **37**, 251-268.

Becker, A., Katzen, F., Pühler, A. & Ielpi, L. (1998). Xanthan gum biosynthesis and application: a biochemical/genetic perspective. *Applied Microbiology and Biotechnology* **50**, 145-152.

Bernier, F. & Berna, A. (2001). Germins and germin-like proteins: Plant do-all proteins. But what do they do exactly? *Plant Physiology and Biochemistry* **39**, 545-554.

Blom, J., Albaum, S. P., Doppmeier, D., Pühler, A., Vorhölter, F. J., Zakrzewski, M. & Goesmann, A. (2009). EDGAR: A software framework for the comparative analysis of prokaryotic genomes. *Bmc Bioinformatics* **10**, 14.

Bogdanove, A., Schornack, S. & Lahaye, T. (2010). TAL effectors: finding plant genes for disease and defense. *Current Opinion in Plant Biology* **13**, 394-401.

Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., Coplin, D. L., Cornelis, G. R., Huang, H. C., Hutcheson, S. W., Panopoulos, N. J. & VanGijsegem, F. (1996). Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. *Molecular Microbiology* **20**, 681-683.

Bolstad, B. M., Irizarry, R. A., Astrand, M. & Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185-193.

Bonshtien, A., Lev, A., Gibly, A., Debbie, P., Avni, A. & Sessa, G. (2005). Molecular properties of the *Xanthomonas* AvrRxv effector and global transcriptional changes determined by its expression in resistant tomato plants. *Molecular Plant-Microbe Interactions* **18**, 300-310.

Bozso, Z., Maunoury, N., Szatmari, A., Mergaert, P., Ott, P. G., Zsiros, L. R., Szabo, E., Kondorosi, E. & Klement, Z. (2009). Transcriptome analysis of a bacterially induced basal and hypersensitive response of *Medicago truncatula*. *Plant Molecular Biology* **70**, 627-646.

Brummer, C. E. (1999). Capturing heterosis in forage crop cultivar development. *Crop Sci* **39**, 943-954.

Brunings, A. M. & Gabriel, D. W. (2003). *Xanthomonas citri*: breaking the surface. *Mol Plant Pathol* **4**, 141-157.

Brunings, A. M., Datnoff, L. E., Ma, J. F., Mitani, N., Nagamura, Y., Rathinasabapathi, B. & Kirst, M. (2009). Differential gene expression of rice in response to silicon and rice blast fungus *Magnaporthe oryzae*. *Annals of Applied Biology* **155**, 161-170.

Büschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Töpsch, S., Vos, P., Salamini, F. & Schulze-Lefert, P. (1997). The barley *Mlo* gene: A novel control element of plant pathogen resistance *Cell* **88**, 695-705.

Büttner, D. & Bonas, U. (2009). Regulation and secretion of *Xanthomonas* virulence factors. *Fems Microbiology Reviews* **34**, 107-133.

Büttner, D. & He, S. Y. (2009). Type III protein secretion in plant pathogenic bacteria. *Plant Physiology* **150**, 1656-1664.

Büttner, D. & Bonas, U. (2010). Regulation and secretion of *Xanthomonas* virulence factors. *Fems Microbiology Reviews* **34**, 107-133.

Büttner, D., and Bonas, U. (2002). Getting across-bacterial type III effector proteins on their way to the plant cell. *The EMBO Journal* **21**, 5313-5322.

Burstein, D., Zusman, T., Degtyar, E., Viner, R., Segal, G. & Pupko, T. (2009). Genome-scale identification of *Legionella pneumophila* effectors using a machine learning approach. *PLoS Pathogens* **5**.

Burton, G. W. (1992). Recurrent restricted phenotypic selection. In *Plant breeding reviews*, pp. 101-113.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S. & Dong, X. N. (1997). The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88**, 57-63.

Carrillo, M. G. C., Goodwin, P. H., Leach, J. E., Leung, H. & Vera Cruz, C. M. (2009). Phylogenomic relationships of rice oxalate oxidases to the cupin superfamily and their association with disease resistance QTL. *Rice* **2**, 67-79.

Catalan, P., Torrecilla, P., Rodriguez, J. A. L. & Olmstead, R. G. (2004). Phylogeny of the festucoid grasses of subtribe *Loliinae* and allies (*Poeae, Pooideae*) inferred from ITS and trnL-F sequences. *Molecular Phylogenetics and Evolution* **31**, 517-541.

Century, K. S., Lagman, R. A., Adkisson, M., Morlan, J., Tobias, R., Schwartz, K., Smith, A., Love, J., Ronald, P. C. & Whalen, M. C. (1999). Developmental control of Xa21-mediated disease resistance in rice. *Plant Journal* **20**, 231-236.

Cernadas, R. A., Camillo, L. R. & Benedetti, C. E. (2008). Transcriptional analysis of the sweet orange interaction with the citrus canker pathogens *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas axonopodis* pv. *aurantifolii*. *Mol Plant Pathol* **9**, 609-631.

Chan, J. W. Y. F. & Goodwin, P. H. (1999). The molecular genetics of virulence of *Xanthomonas campestris*. *Biotechnology Advances* **17**, 489-508.

Chen, X. W., Hackett, C. A., Niks, R. E., Hedley, P. E., Booth, C., Druka, A., Marcel, T. C., Vels, A., Bayer, M., Milne, I., Morris, J., Ramsay, L., Marshall, D., Cardle, L. & Waugh, R. (2010). An eQTL analysis of partial resistance to *Puccinia hordei* in barley. *PLoS One* **5**.

Cho, H. J., Park, Y. J., Noh, T. H., Kim, Y. T., Kim, J. G., Song, E. S., Lee, D. H. & Lee, B. M. (2008). Molecular analysis of the hrp gene cluster in *Xanthomonas oryzae* pathovar *oryzae* KACC10859. *Microbial Pathogenesis* **44**, 473-483.

Christensen, A. B., Thordal-Christensen, H., Zimmermann, G., Gjetting, T., Lyngkjaer, M. F., Dudler, R. & Schweizer, P. (2004). The germinlike protein GLP4 exhibits superoxide dismutase activity and is an important component of quantitative resistance in wheat and barley. *Molecular Plant-Microbe Interactions* **17**, 109-117.

Collard, B. C. Y. & Mackill, D. J. (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B-Biological Sciences* **363**, 557-572.

Collins, A., Milbourne, D., Ramsay, L., Meyer, R., Chatot-Balandras, C., Oberhagemann, P., De Jong, W., Gebhardt, C., Bonnel, E. & Waugh, R. (1999). QTL for field resistance to late blight in potato are strongly correlated with maturity and vigour. *Molecular Breeding* **5**, 387-398.

Collins, N. C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J. L., Huckelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S. C. & Schulze-Lefert, P. (2003). SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* **425**, 973-977.

Cornelis, G. R. & Van Gijsegem, F. (2000). Assembly and function of type III secretory systems. *Annual Review Of Microbiology* **54**, 735-774.

Cornelis, G. R. (2006). The type III secretion injectisome. *Nature Reviews Microbiology* **4**, 811-825.

Da Silva, A. C. R., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Sluys, M. A., Almeida, N. F., Alves, L. M. C., Do Amaral, A. M., Bertolini, M. C., Camargo, L. E. A., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Clapina, L. P., Cicarelli, R. M. B., Coutinho, L. L., Cursino-Santos, J. R., El-Dorry, H., Faria, J. B., Ferreira, A. J. S., Ferreira, R. C. C., Ferro, M. I. T., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G. M., Lemos, M. V. F., Locali, E. C., Machado, M. A., Madeira, A., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F. M., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T. M., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A. D., Silva, C., De Souza, R. F., Spinola, L. A. F., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I. D., Dos Santos, M. T., Truffi, D., Tsai, S. M., White, F. F., Setubal, J. C. & Kitajima, J. P. (2002). Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* **417**, 459-463.

Danesh, D., Aarons, S., McGill, G. E. & Young, N. D. (1994). Genetic dissection of oligogenic resistance to bacterial wilt in tomato. *Molecular Plant-Microbe Interactions* **7**, 464-471.

Dangl, J. L. & Jones, J. D. G. (2001). Plant pathogens and integrated defence responses to infection. *Nature* **411**, 826-833.

Darsonval, A., Darrasse, A., Meyer, D., Demarty, M., Durand, K., Bureau, C., Manceau, C. & Jacques, M. A. (2008). The type III secretion system of *Xanthomonas fuscans* subsp *fuscans* is involved in the phyllosphere colonization process and in transmission to seeds of susceptible beans. *Applied and Environmental Microbiology* **74**, 2669-2678.

De Mendiburu, F. (2009). agricolae: Statistical Procedures for Agricultural Research. *R package version 10-7*.

Delgado, C. L. (2005). Rising demand for meat and milk in developing countries: implications for grasslands-based livestock production. In *Grassland: A Global Resource*, pp. 29-39. Edited by D. A. McGilloway.

Devos, K. M. (2005). Updating the 'crop circle'. *Current Opinion In Plant Biology* **8**, 155-162.

Deyholos, M. K. (2010). Making the most of drought and salinity transcriptomics. *Plant Cell Environ* **33**, 648-654.

Dharmapuri, S. & Sonti, R. V. (1999). A transposon insertion in the gumG homologue of *Xanthomonas oryzae* pv. *oryzae* causes loss of extracellular polysaccharide production and virulence. *FEMS Microbiol Lett* **179**, 53-59.

Dobrindt, U., Hochhut, B., Hentschel, U. & Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. *Nature Reviews Microbiology* **2**, 414-424.

Dow, J. M., Osbourn, A. E., Wilson, T. J. G. & Daniels, M. J. (1995). A locus determining pathogenicity of *Xanthomonas campestris* is involved in lipopolysaccharide biosynthesis. *Molecular Plant-Microbe Interactions* **8**, 768-777.

Dow, J. M., Feng, J. X., Barber, C. E., Tang, J. L. & Daniels, M. J. (2000). Novel genes involved in the regulation of pathogenicity factor production within the rpf gene cluster of *Xanthomonas campestris*. *Microbiology* **146**, 885-891.

Dow, J. M., Crossman, L., Findlay, K., He, Y. Q., Feng, J. X. & Tang, J. L. (2003). Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10995-11000.

Dracatos, P. M., Cogan, N. O. I., Sawbridge, T. I., Gendall, A. R., Smith, K. F., Spangenberg, G. C. & Forster, J. W. (2009). Molecular characterisation and genetic mapping of candidate genes for qualitative disease resistance in perennial ryegrass (*Lolium perenne* L.). *BMC Plant Biology* **9**.

Dracatos, P. M., Cogan, N. O. I., Keane, P. J., Smith, K. F. & Forster, J. W. (2010). Biology and genetics of crown rust disease in ryegrasses. *Crop Sci* **50**, 1605-1624.

Dumsday, J. L., Smith, K. F., Forster, J. W. & Jones, E. S. (2003). SSR based genetic linkage analysis of resistance to crown rust (*Puccinia coronata* f. sp. *lolii*) in perennial ryegrass (*Lolium perenne*). *Plant Pathol* **52**, 628-637.

Dunger, G., Relling, V. M., Tondo, M. L., Barreras, M., Ielpi, L., Orellano, E. G. & Ottado, J. (2007). Xanthan is not essential for pathogenicity in citrus canker but contributes to *Xanthomonas* epiphytic survival. *Archives of Microbiology* **188**, 127-135.

Dunwell, J. M., Gibbings, J. G., Mahmood, T. & Naqvi, S. M. S. (2008). Germin and germin-like proteins: Evolution, structure, and function. *Critical Reviews in Plant Sciences* **27**, 342-375.

Durrant, W. E., Rowland, O., Piedras, P., Hammond-Kosack, K. E. & Jones, J. D. G. (2000). cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell* **12**, 963-977.

Dye, D. W. & Lelliott, R. A. (1974). Genus 11. *Xanthomonas*. In *Bergey's manual of determinative bacteriology*, pp. 243-249. Edited by R. E. Buchan & N. E. Gibbows. Baltimore, MD: Williams & Wilkins.

Egli, T., Goto, M. & Schmidt, D. (1975). Bacterial wilt, a new forage grass disease. *Phytopathologische Zeitschrift* **82**, 111-121.

Egli, T. & Schmidt, D. (1982). Pathogenic variation among the causal agents of baterial wilt of forage grasses. *Phytopathologische Zeitschrift* **104**, 138-150.

El Attari, H., Sarrafi, A., Alizadeh, A., Dechamp-Guillaume, G. & Barrault, G. (1996). Genetic analysis of partial resistance to bacterial leaf streak (*Xanthomonas campestris* pv. *cerealis*) in wheat. *Plant Pathol* **45**, 736-741.

Epstein, E. (2009). Silicon: its manifold roles in plants. *Annals of Applied Biology* **155**, 155-160.

Excoffier, L., Smouse, P. E. & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479-491.

Faris, J. D., Li, W. L., Liu, D. J., Chen, P. D. & Gill, B. S. (1999). Candidate gene analysis of quantitative disease resistance in wheat. *Theoretical and Applied Genetics* **98**, 219-225.

Fauteux, F., Chain, F., Belzile, F., Menzies, J. G. & Belanger, R. R. (2006). The protective role of silicon in the Arabidopsis-powdery mildew pathosystem. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 17554-17559.

Fenselau, S. & Bonas, U. (1995). Sequence and expression analysis of the *hrpB* pathogenicity operon of *Xanthomonas campestris* pv. *vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems. *Molecular Plant-Microbe Interactions* **8**, 845-854.

Flint-Garcia, S. A., Darrah, L. L., McMullen, M. D. & Hibbard, B. E. (2003). Phenotypic versus marker-assisted selection for stalk strength and second-generation European corn borer resistance in maize. *Theoretical and Applied Genetics* **107**, 1331-1336.

Flor, H. H. (1971). Current status of gene-for-gene concept. *Annual Review of Phytopathology* **9**, 275-&.

Fodor, S. P. A., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T. & Solas, D. (1991). Light-directed, spatially addressable parallel chemical synthesis. *Science* **251**, 767-773.

Francia, E., Tacconi, G., Crosatti, C., Barabaschi, D., Bulgarelli, D., Dall'Aglio, E. & Vale, G. (2005). Marker assisted selection in crop plants. *Plant Cell Tissue And Organ Culture* **82**, 317-342.

Furutani, A., Takaoka, M., Sanada, H., Noguchi, Y., Oku, T., Tsuno, K., Ochiai, H. & Tsuge, S. (2009). Identification of Novel Type III Secretion Effectors in *Xanthomonas oryzae* pv. *oryzae*. *Molecular Plant-Microbe Interactions* **22**, 96-106.

Galan, J. E. & Collmer, A. (1999). Type III secretion machines: Bacterial devices for protein delivery into host cells. *Science* **284**, 1322-1328.

Gamas, P., Niebel, F. D. C., Lescure, N. & Cullimore, J. V. (1996). Use of a subtractive hybridization approach to identify new *Medicago truncatula* genes induced during root nodule development. *Molecular Plant-Microbe Interactions* **9**, 233-242.

Gaut, B. S. (2002). Evolutionary dynamics of grass genomes. *New Phytologist* **154**, 15-28; many ref.

Geiger, H. H. & Heun, M. (1989). Genetics of quantitative resistance to fungal diseases. *Annual Review of Phytopathology* **27**, 317-341.

Gennaro, A., Koebner, R. M. D. & Ceoloni, C. (2009). A candidate for Lr19, an exotic gene conditioning leaf rust resistance in wheat. *Funct Integr Genomics* **9**, 325-334.

Ghosh, P. (2004). Process of protein transport by the type III secretion system. *Microbiology and Molecular Biology Reviews* **68**, 771-795.

Gibly, A., Bonshtien, A., Balaji, V., Debbie, P., Martin, G. B. & Sessa, G. (2004). Identification and expression profiling of tomato genes differentially regulated during a resistance response to *Xanthomonas campestris* pv. *vesicatoria*. *Molecular Plant-Microbe Interactions* **17**, 1212-1222.

Gilad, Y., Oshlack, A., Smyth, G. K., Speed, T. P. & White, K. P. (2006). Expression profiling in primates reveals a rapid evolution of human transcription factors. *Nature* **440**, 242-245.

Gill, G. P., Wilcox, P. L., Whittaker, D. J., Winz, R. A., Bickerstaff, P., Echt, C. E., Kent, J., Humphreys, M. O., Elborough, K. M. & Gardner, R. C. (2006). A framework linkage map of perennial ryegrass based on SSR markers. *Genome* **49**, 354-364.

Gilliland, T. J., Coll, R., Calsyn, E., De Loose, M., van Eijk, M. J. T. & Roldán-Ruiz, I. (2000). Estimating genetic conformity between related ryegrass (*Lolium*) varieties. II. Morphology and biochemical characterisation. *Molecular Breeding* **6**, 569-580.

Gollob, H. F. (1968). A statistical model which combines features of factor analytic and analysis of variance techniques. *Psychometrika* **33**, 73-114.

Gophna, U., Ron, E. Z. & Graur, D. (2003). Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. *Gene* **312**, 151-163.

Gregersen, P. L., Brinch-Pedersen, H. & Holm, P. B. (2005). A microarray-based comparative analysis of gene expression profiles during grain development in transgenic and wild type wheat. *Transgenic Research* **14**, 887-905.

Gudesblat, G. E., Torres, P. S. & Vojnov, A. A. (2009). *Xanthomonas campestris* overcomes *Arabidopsis* stomatal innate immunity trough a DSF cell-to-cell signal-regulated virulence factor. *Plant Physiology* **149**, 1017-1027.

Gürlebeck, D., Thieme, F. & Bonas, U. (2006). Type III effector proteins from the plant pathogen *Xanthomonas* and their role in the interaction with the host plant. *Journal of Plant Physiology* **163**, 233-255.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95-98.

Hammond-Kosack, K. E. & Jones, J. D. G. (1997). Plant disease resistance genes. *Annual Review of Phytopathology* **48**, 575-607.

Hashimi, S. M., Wall, M. K., Smith, A. B., Maxwell, A. & Birch, R. G. (2007). The phytotoxin albicidin is a novel inhibitor of DNA gyrase. *Antimicrobial Agents and Chemotherapy* **51**, 181-187.

Hastie, T. & Efron, B. (2007). lars: least angle regression, lasso and forward stagewise. *R package version 09-7*.

Hauben, L., Vauterin, L., Swings, J. & Moore, E. R. B. (1997). Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *International Journal of Systematic Bacteriology* **47**, 328-335.

Hauser, B. A., Pratt, L. H. & CordonnierPratt, M. M. (1997). Absolute quantification of five phytochrome transcripts in seedlings and mature plants of tomato (*Solanum lycopersicum* L). *Planta* **201**, 379-387.

Hayward, A. C. (1993). The hosts of *Xanthomonas*. In *Xanthomonas*, pp. 1-119. Edited by J. G. Swings & E. L. Civerolo. London: Chapman & Hall.

Hirata, M., Cai, H., Inoue, M., Yuyama, N., Miura, Y., Komatsu, T., Takamizo, T. & Fujimori, M. (2006). Development of simple sequence repeat (SSR) markers and construction of an SSR-based linkage map in Italian ryegrass (*Lolium multiflorum* Lam.). *Theoretical and Applied Genetics* **113**, 270.

Hogenhout, S. A., Van der Hoorn, R. A. L., Terauchi, R. & Kamoun, S. (2009). Emerging concepts in effector biology of plant-associated organisms. *Molecular Plant-Microbe Interactions* **22**, 115-122.

Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* **6**, 65-70.

Holt, R. A. & Jones, S. J. M. (2008). The new paradigm of flow cell sequencing. *Genome Research* **18**, 839-846.

Horton, R. M. (1995). PCR-Mediated recombination and mutagenesis. *Molecular Biotechnology* **3**, 93-99.

Hothorn, T., Hornik, K., Van de Wiel, M. A. & Zeileis, A. (2006). A lego system for conditional inference. *The American Statistician* **60**, 257-263.

Hotson, A., Chosed, R., Shu, H. J., Orth, K. & Mudgett, M. B. (2003). *Xanthomonas* type III effector XopD targets SUMO-conjugated proteins in planta. *Molecular Microbiology* **50**, 377-389.

Hua, J., Zhang, Y., Qian, W. & He, C. Z. (2007). Avirulence gene and insertion element-based RFLP as well as RAPD markers reveal high levels of genomic polymorphism in the rice pathogen *Xanthomonas oryzae* pv. *oryzae*. *Systematic and Applied Microbiology* **30**, 587-600.

Huang, N., Angeles, E. R., Domingo, J., G., M., Singh, S., Zhang, G., Kumaravadivel, N., Bennett, J. & Khush, G. S. (1997). Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. *Theoretical and Applied Genetics* **95**, 313-320.

Huguet, E., Hahn, K., Wengelnik, K. & Bonas, U. (1998). *hpaA* mutants of *Xanthomonas campestris* pv. *vesicatoria* are affected in pathogenicity but retain the ability to induce host-specific hypersensitive reaction. *Molecular Microbiology* **29**, 1379-1390.

Humphreys, M., Feuerstein, U., Vandevalle, M. & Baert, J. (2010). Ryegrasses. In *Fodder crops and amenity grasses*, pp. 211-260. Edited by B. Boller, U. K. Posselt & F. Veronesi: Springer.

Humphreys, M. O. (2005). Genetic improvement of forage crops - past, present and future. *Journal of Agricultural Science* **143**, 441-448.

Humphry, M., Consonni, C. & Panstruga, R. (2006). mlo-based powdery mildew immunity: silver bullet or simply non-host resistance? *Mol Plant Pathol* **7**, 605-610.

Ikeda, S. (2005). Isolation of disease resistance gene analogs from Italian Ryegrass (*Lolium multiflorum* Lam.). *Grassland Science* **51**, 63-70.

Imaizumi, S., Honda, M. & Fujimori, T. (1999). Effect of temperature on the control of annual bluegrass (*Poa annua* L.) With *Xanthomonas campestris* pv. *poae* (Jt-P482). *Biological Control* **16**, 13-17.

Inoue, M., Gao, Z., Hirata, M., Fujimori, M. & Cai, H. (2004). Construction of a high-density linkage map of Italian ryegrass (*Lolium multiflorum* Lam.) using restriction fragment length polymorphism, amplified fragment length polymorphism, and telomeric repeat associated sequence markers. *Genome* **47**, 57-65.

Iyer, A. S. & McCouch, S. R. (2004). The rice bacterial blight resistance gene *xa5* encodes a novel form of disease resistance. *Molecular Plant-Microbe Interactions* **17**, 1348-1354.

Jiang, B., Xu, R. Q., Li, X. Z., Wei, H. Y., Bai, F., Hu, X., He, Y. Q. & Tang, J. L. (2006a). Construction and characterization of a *hrpG* mutant rendering constitutive expression of *hrp* genes in *Xanthomonas campestris* pv. *campestris*. *Progress in Natural Science* **16**, 480-485.

Jiang, G.-H., Xia, Z.-H., Zhou, Y.-L., Wan, J., Li, D.-Y., Chen, R.-S., Zhai, W.-X. & Zhu, L.-H. (2006b). Testifying the rice bacterial blight resistance gene *xa5* by genetic complementation and further analyzing *xa5* (*Xa5*) in comparison with its homolog *TFIIAγ1*. *Molecular Genetics and Genomics* **275**, 354.

Jones, E. S., Mohoney, N. L., Hayward, M. D., Armstead, I. P., Jones, J. G., Humphreys, M. O., King, I. P., Kishida, T., Yamada, T., Balfourier, F., Charmet, G. & Forster, J. W. (2002). An enhanced molecular marker based genetic map of perennial ryegrass (*Lolium perenne*) reveals comparative relationships with other *Poaceae* genomes. *Genome* **45**, 282-295.

Jones, J. D. G. & Dangl, J. L. (2006). The plant immune system. *Nature* **444**, 323-329.

Kaniga, K., Delor, I. & Cornelis, G. R. (1991). A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria - inactivation of the *blaA* Gene of *Yersinia enterocolitica*. *Gene* **109**, 137-141.

Katzen, F., Ferreiro, D. U., Oddo, C. G., Ielmini, M. V., Becker, A., Pühler, A. & Ielpi, L. (1998). *Xanthomonas campestris* pv. *campestris* gum mutants: Effects on xanthan biosynthesis and plant virulence. *Journal Of Bacteriology* **180**, 1607-1617.

Kay, S. & Bonas, U. (2009). How *Xanthomonas* type III effectors manipulate the host plant. *Current Opinion in Microbiology* **12**, 37-43.

Kemp, B. P., Horne, J., Bryant, A. & Cooper, R. M. (2004). *Xanthomonas axonopodis* pv. *manihotis gumD* gene is essential, for EPS production and pathogenicity and enhances epiphytic survival on cassava (*Manihot esculenta*). *Physiological and Molecular Plant Pathology* **64**, 209-218.

Kim, H. J., Pesacreta, T. C. & Triplett, B. A. (2004). Cotton-fiber germin-like protein. II: Immunolocalization, purification, and functional analysis. *Planta* **218**, 525-535.

Kim, J. G., Taylor, K. W., Hotson, A., Keegan, M., Schmelz, E. A. & Mudgett, M. B. (2008). XopD SUMO protease affects host transcription, promotes pathogen growth, and delays symptom development in *Xanthomonas*-infected tomato leaves. *Plant Cell* **20**, 1915-1929.

Kim, J. G., Li, X. Y., Roden, J. A., Taylor, K. W., Aakre, C. D., Su, B., Lalonde, S., Kirik, A., Chen, Y. H., Baranage, G., McLane, H., Martin, G. B. & Mudgett, M. B. (2009). *Xanthomonas* T3S effector XopN suppresses PAMP-triggered immunity and interacts with a tomato atypical receptor-like kinase and TFT1. *Plant Cell* **21**, 1305-1323.

Kimbeng, C. A. (1999). Genetic basis of crown rust resistance in perennial ryegrass, breeding strategies, and genetic variation among pathogen populations: a review. *Australian Journal of Experimental Agriculture* **39**, 361-378.

Kingsley, M. T., Gabriel, D. W., Marlow, G. C. & Roberts, P. D. (1993). The *opsX* locus of *Xanthomonas campestris* affects host-range and biosynthesis of lipopolysaccharide and extracelluar polysaccharide. *Journal of Bacteriology* **175**, 5839-5850.

Kjemtrup, S., Nimchuk, Z. & Dangl, J. L. (2000). Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. *Current Opinion in Microbiology* **3**, 73-78.

Kölliker, R., Stadelmann, F. J., Reidy, B. & Nösberger, J. (1999). Genetic variability of forage grass cultivars: A comparison of *Festuca pratensis* Huds., *Lolium perenne* L. and *Dactylis glomerata* L. *Euphytica* **106**, 261-270.

Kölliker, R., Kraehenbuehl, R., Boller, B. & Widmer, F. (2006). Genetic diversity and pathogenicity of the grass pathogen *Xanthomonas translucens* pv. *graminis*. *Systematic and Applied Microbiology* **29**, 109-119.

Kopecky, D., Bartos, J., Lukaszewski, A. J., Baird, J. H., Cernoch, V., Kölliker, R., Rognli, O. A., Blois, H., Caig, V., Lübberstedt, T., Studer, B., Shaw, P., Dolezel, J. & Kilian, A. (2009). Development and mapping of DArT markers within the *Festuca-Lolium* complex. *BMC Genomics* **10**.

Kottapalli, K. R., Satoh, K., Rakwal, R., Shibato, J., Doi, K., Nagata, T. & Kikuchi, S. (2007). Combining in silico mapping and arraying: an approach to identifying common candidate genes for submergence tolerance and resistance to bacterial leaf blight in ice. *Molecules and Cells* **24**, 394-408.

Kvedaras, O. L., Byrne, M. J., Coombes, N. E. & Keeping, M. G. (2009). Influence of plant silicon and sugarcane cultivar on mandibular wear in the stalk borer *Eldana saccharina*. *Agricultural and Forest Entomology* **11**, 301-306.

Lane, B. G. (1994). Oxalate, germin, and the extracellular matirx of higher plants. *Faseb Journal* **8**, 294-301.

Langridge, P., Lagudah, E. S., Holton, T. A., Appels, R., Sharp, P. J. & Chalmers, K. J. (2001). Trends in genetic and genome analyses in wheat: a review. *Australian Journal of Agricultural Research* **52**, 1043-1077.

Leach, J. E. & White, F. F. (1996). Bacterial avirulence genes. *Annual Review of Phytopathology* **34**, 153-179.

Leach, J. E., Vera Cruz, C. M., Bai, J. & Leung, H. (2001). Pathogen fitness penalty as a predictor of durability of disease resistance genes. *Annual Review of Phytopathology* **39**, 187-224.

Lee, B. M., Park, Y. J., Park, D. S., Kang, H. W., Kim, J. G., Song, E. S., Park, I. C., Yoon, U. H., Hahn, J. H., Koo, B. S., Lee, G. B., Kim, H., Park, H. S., Yoon, K. O., Kim, J. H., Jung, C., Koh, N. H., Seo, J. S. & Go, S. J. (2005). The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice. *Nucleic Acids Research* **33**, 577-586.

Lee, S. W., Han, S. W., Bartley, L. E. & Ronald, P. C. (2006). Unique characteristics of *Xanthomonas oryzae* pv. *oryzae* AvrXa21 and implications for plant innate immunity. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 18395-18400.

Lee, S. W., Jeong, K. S., Han, S. W., Lee, S. E., Phee, B. K., Hahn, T. R. & Ronald, P. (2008). The *Xanthomonas oryzae* pv. *oryzae* PhoPQ two-component system is required for AvrXA21 activity, hrpG expression, and virulence. *Journal of Bacteriology* **190**, 2183-2197.

Leyns, F., Decleene, M., Swings, J. G. & Deley, J. (1984). The host range of the genus *Xanthomonas*. *Botanical Review* **50**, 308-356.

Leyns, F., De Cleene, M., Van Bogaert, G., Van de Wiele, A. & De Ley, J. (1988). Preliminary investigations about the mode of transmission and spread of *Xanthomonas campestris* pv. *graminis* on forage grasses. *Journal of Phytopathology* **122**, 76-88.

Leyns, F. (1993). *Xanthomonas campestris* pv. *graminis:* Cause of bacterial wilt of forage grasses. In *Xanthomonas*, pp. 55-57. Edited by J. G. Swings & E. L. Civerolo. London: Chapman & Hall.

Li, J., Xu, Y. H., Fei, S. Z. & Li, L. J. (2006a). Isolation, characterization and evolutionary analysis of resistance gene analogs in annual ryegrass, perennial ryegrass and their hybrid. *Physiologia Plantarum* **126**, 627-638.

Li, Q., Chen, F., Sun, L. X., Zhang, Z. Q., Yang, Y. N. & He, Z. H. (2006b). Expression profiling of rice genes in early defense responses to blast and bacterial blight pathogens using cDNA microarray. *Physiological and Molecular Plant Pathology* **68**, 51-60.

Liang, P. & Pardee, A. B. (1992). Differential display of eukaryotid messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.

Liang, Y. C., Sun, W. C., Zhu, Y. G. & Christie, P. (2007). Mechanisms of silicon-mediated alleviation of abiotic stresses in higher plants: A review. *Environmental Pollution* **147**, 422-428.

Lipshutz, R. J., Fodor, S. P. A., Gingeras, T. R. & Lockhart, D. J. (1999). High density synthetic oligonucleotide arrays. *Nature Genetics* **21**, 20-24.

Liu, B., Zhang, S. H., Zhu, X. Y., Yang, Q. Y., Wu, S. Z., Mei, M. T., Mauleon, R., Leach, J., Mew, T. & Leung, H. (2004). Candidate defense genes as predictors of quantitative blast resistance in rice. *Molecular Plant-Microbe Interactions* **17**, 1146-1152.

Liu, S. X. & Anderson, J. A. (2003). Marker assisted evaluation of *Fusarium* head blight resistant wheat germplasm. *Crop Sci* **43**, 760-766.

Lonnstedt, I. & Speed, T. (2002). Replicated microarray data. *Statistica Sinica* **12**, 31-46.

Löwer, M. & Schneider, G. (2009). Prediction of Type III Secretion Signals in Genomes of Gram-Negative Bacteria. *PLoS One* **4**, 9.

Lu, H., Patil, P., Van Sluys, M. A., White, F. F., Ryan, R. P., Dow, J. M., Rabinowicz, P., Salzberg, S. L., Leach, J. E., Sonti, R., Brendel, V. & Bogdanove, A. J. (2008). Acquisition and evolution of plant pathogenesis-associated gene clusters and candidate determinants of tissue-specificity in *Xanthomonas*. *PLoS One* **3**.

Ma, J. F. & Yamaji, N. (2006). Silicon uptake and accumulation in higher plants. *Trends in Plant Science* **11**, 392-397.

Macnab, R. M. (2003). How bacteria assemble flagella. *Annual Review of Microbiology* **57**, 77-100.

Manosalva, P. M., Davidson, R. M., Liu, B., Zhu, X. Y., Hulbert, S. H., Leung, H. & Leach, J. E. (2009). A Germin-Like Protein Gene Family Functions as a Complex Quantitative Trait Locus Conferring Broad-Spectrum Disease Resistance in Rice. *Plant Physiology* **149**, 286-296.

Mansfield, J., Jenner, C., Hockenhull, R., Bennett, M. A. & Stewart, R. (1994). Characterization of *avrPphE*, a gene for cultivar-specific avirulence from *Pseudomonas syringae* pv. *phaseolicola* which is physically linked to *hrpY*, an new *hrp* gene identified in the halo-blight bacterium. *Molecular Plant-Microbe Interactions* **7**, 726-739.

Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., Berka, J., Braverman, M. S., Chen, Y. J., Chen, Z. T., Dewell, S. B., Du, L., Fierro, J. M., Gomes, X. V., Godwin, B. C., He, W., Helgesen, S., Ho, C. H., Irzyk, G. P., Jando, S. C., Alenquer, M. L. I., Jarvie, T. P., Jirage, K. B., Kim, J. B., Knight, J. R., Lanza, J. R., Leamon, J. H., Lefkowitz, S. M., Lei, M., Li, J., Lohman, K. L., Lu, H., Makhijani, V. B.,

McDade, K. E., McKenna, M. P., Myers, E. W., Nickerson, E., Nobile, J. R., Plant, R., Puc, B. P., Ronan, M. T., Roth, G. T., Sarkis, G. J., Simons, J. F., Simpson, J. W., Srinivasan, M., Tartaro, K. R., Tomasz, A., Vogt, K. A., Volkmer, G. A., Wang, S. H., Wang, Y., Weiner, M. P., Yu, P. G., Begley, R. F. & Rothberg, J. M. (2005). Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376-380.

Martin, R. C., Hollenbeck, V. G. & Dombrowski, J. E. (2008). Evaluation of reference genes for quantitative RT-PCR in *Lolium perenne*. *Crop Sci* **48**, 1881-1887.

McDermott, J. E., Corrigan, A., Peterson, E., Oehmen, C., Niemann, G., Cambronne, E. D., Sharp, D., Adkins, J. N., Samudrala, R. & Heffron, F. (2011). Computational prediction of type III and IV secreted effectors in Gram-negative bacteria *Infection and Immunity* **79**, 23-32.

McDonald, B. A. & Linde, C. (2002). Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology* **40**, 349-379.

Metz, M., Dahlbeck, D., Morales, C. Q., Al Sady, B., Clark, E. T. & Staskawicz, B. J. (2005). The conserved *Xanthomonas campestris* pv. *vesicatoria* effector protein XopX is a virulence factor and suppresses host defense in *Nicotiana benthamiana*. *Plant Journal* **41**, 801-814.

Meyer, F., Goesmann, A., McHardy, A. C., Bartels, D., Bekel, T., Clausen, J., Kalinowski, J., Linke, B., Rupp, O., Giegerich, R. & Pühler, A. (2003). GenDB - an open source genome annotation system for prokaryote genomes. *Nucleic Acids Research* **31**, 2187-2195.

Michel, V. V. (2001). Interactions between *Xanthomonas campestris* pv. *graminis* strains and meadow fescue and Italian rye grass cultivars. *Plant Disease* **85**, 538-542.

Miller, J. R., Koren, S. & Sutton, G. (2010). Assembly algorithms for next-generation sequencing data. *Genomics* **95**, 315-327.

Miller, V. L. & Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations - osmoregulation of outer-membrane proteins and virulence determinants in *Vibrio cholerae* requires Toxr. *Journal of Bacteriology* **170**, 2575-2583.

Minsavage, G. V., Dahlbeck, D., Whalen, M. C., Kearney, B., Bonas, U., Staskawicz, B. J. & Stall, R. E. (1990). Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. *vesicatoria*-pepper interactions. *Molecular Plant-Microbe Interactions* **3**, 41-47.

Moore, S., Payton, P., Wright, M., Tanksley, S. & Giovannoni, J. (2005). Utilization of tomato microarrays for comparative gene expression analysis in the Solanaceae. *Journal of Experimental Botany* **56**, 2885-2895.

Moscou, M. J. & Bogdanove, A. J. (2009). A simple cipher governs DNA recognition by TAL effectors. *Science* **326**, 1501-1501.

Mudgett, M. B., Chesnokova, O., Dahlbeck, D., Clark, E. T., Rossier, O., Bonas, U. & Staskawicz, B. J. (2000). Molecular signals required for type III secretion and translocation of the *Xanthomonas campestris* AvrBs2 protein to pepper plants. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13324-13329.

Müller-Hug, B. (2008). Race-specificity and *in planta* detection of *Xanthomonas translucens* pv. *graminis* in *Lolium multiflorum*. In *Agroscope Reckenholz-Tänikon Reasearch Station ART*, pp. 101. Zürich: Zürich

Mukaihara, T., Tamura, N. & Iwabuchi, M. (2010). Genome-wide identification of a large repertoire of *Ralstonia solanacearum* type III effector proteins by a new functional screen. *Molecular Plant-Microbe Interactions* **23**, 251-262.

Mukherjee, S., Hao, Y. H. & Orth, K. (2007). A newly discovered post-translational modification - the acetylation of serine and threonine residues. *Trends in Biochemical Sciences* **32**, 210-216.

Mutlu, N., Miklas, P., Reiser, J. & Coyne, D. (2005). Backcross breeding for improved resistance to common bacterial blight in pinto bean (*Phaseolus vulgaris* L.). *Plant Breed* **124**, 282-287.

Mutlu, N., Miklas, P. & Coyne, D. (2006). Resistance gene analog polymorphism (RGAP) markers co-localize with disease resistance genes and QTL in common bean. *Molecular Breeding* **17**, 127.

Mysore, K. S., D'Ascenzo, M. D., He, X. H. & Martin, G. B. (2003). Overexpression of the disease resistance gene *Pto* in tomato induces gene expression changes similar to immune responses in human and fruitfly. *Plant Physiology* **132**, 1901-1912.

Nayak, D., Bose, L. K., Singh, S. & Nayak, P. (2008). Additive main effects and multiplicative interaction analysis of host-pathogen relationship in rice-bacterial blight pathosystem. *Plant Pathology Journal* **24**, 337-351.

Newman, M. A., Dow, J. M. & Daniels, M. J. (2001). Bacterial lipopolysaccharides and plant-pathogen interactions. *European Journal of Plant Pathology* **107**, 95-102.

Niño-Liu, D. O., Ronald, P. C. & Bogdanove, A. J. (2006). *Xanthomonas oryzae* pathovars: model pathogens of a model crop. *Mol Plant Pathol* **7**, 303-324.

Noel, L., Thieme, F., Nennstiel, D. & Bonas, U. (2001). cDNA-AFLP analysis unravels a genome-wide HrpG-regulon in the plant pathogen *Xanthomonas campestris* pv. *vesicatoria*. *Molecular Microbiology* **41**, 1271-1281.

Noel, L., Thieme, F., Nennstiel, D. & Bonas, U. (2002). Two novel type III-secreted proteins of *Xanthomonas campestris* pv. *vesicatoria* are encoded within the hrp pathogenicity island. *Journal of Bacteriology* **184**, 1340-1348.

Novatchkova, M., Budhiraja, R., Coupland, G., Eisenhaber, F. & Bachmair, A. (2004). SUMO conjugation in plants. *Planta* **220**, 1-8.

Ochiai, H., Inoue, V., Takeya, M., Sasaki, A. & Kaku, H. (2005). Genome sequence of *Xanthomonas oryzae* pv. *oryzae* suggests contribution of large numbers of effector genes and insertion sequences to its race diversity. *Jarq-Japan Agricultural Research Quarterly* **39**, 275-287.

Okoniewski, M. J. & Miller, C. J. (2006). Hybridization interactions between probesets in short oligo microarrays lead to spurious correlations. *Bmc Bioinformatics* **7**.

Palmer, T., Sargent, F. & Berks, B. C. (2005). Export of complex cofactor-containing proteins by the bacterial Tat pathway. *Trends in Microbiology* **13**, 175-180.

Pantoja, M., Chen, L. S., Chen, Y. C. & Nester, E. W. (2002). *Agrobacterium* type IV secretion is a two-step process in which export substrates associate with the virulence protein VirJ in the periplasm. *Molecular Microbiology* **45**, 1325-1335.

Paquet, A. & Yang, J. Y. H. (2008).arrayQuality: Assessing array quality on spotted arrays.

Parkinson, N., Cowie, C., Heeney, J. & Stead, D. (2009). Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences. *International Journal of Systematic and Evolutionary Microbiology* **59**, 264-274.

Paul, H. & Smith, I. M. (1989). Bacterial pathogens of gramineae: systematic review and assessment of quarantine status for the EPPO region. *EPPO Bulletin* **19**, 33-42.

Perumalsamy, S., Bharani, M., Sudha, M., Nagarajan, P., Arul, L., Saraswathi, R., Balasubramanian, P. & Ramalingam, J. (2010). Functional marker-assisted selection for bacterial leaf blight resistance genes in rice (*Oryza sativa* L.). *Plant Breed* **129**, 400-406.

Pfaffl, M. W., Horgan, G. W. & Dempfle, L. (2002). Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30**.

Picardi, E., Horner, D. S., Chiara, M., Schiavon, R., Valle, G. & Pesole, G. (2010). Large-scale detection and analysis of RNA editing in grape mtDNA by RNA deep-sequencing. *Nucleic Acids Research* **38**, 4755-4767.

Pieretti, I., Royer, M., Barbe, V., Carrere, S., Koebnik, R., Cociancich, S., Couloux, A., Darrasse, A., Gouzy, J., Jacques, M. A., Lauber, E., Manceau, C., Mangenot, S., Poussier, S., Segurens, B., Szurek, B., Verdier, V., Arlat, M. & Rott, P. (2009). The complete genome sequence of *Xanthomonas albilineans* provides new insights into the reductive genome evolution of the xylem-limited *Xanthomonadaceae*. *BMC Genomics* **10**, 15.

Posselt, U. (2010). Breeding methods in cross-pollinated species. In *Handbook of Plant Breeding: Fodder Crops and Amenity Grasses*, pp. 39-88. Edited by B. Boller, U. Posselt & F. Veronesi. New York: Springer Science + Business Media.

Qian, W., Jia, Y. T., Ren, S. X., He, Y. Q., Feng, J. X., Lu, L. F., Sun, Q. H., Ying, G., Tang, D. J., Tang, H., Wu, W., Hao, P., Wang, L. F., Jiang, B. L., Zeng, S. Y., Gu, W. Y., Lu, G., Rong, L., Tian, Y. C., Yao, Z. J., Fu, G., Chen, B. S., Fang, R. X., Qiang, B. Q., Chen, Z., Zhao, G. P., Tang, J. L. & He, C. Z. (2005). Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris* pv. *campestris*. *Genome Research* **15**, 757-767.

Qin, L., Overmars, B., Helder, J., Popeijus, H., van der Voort, J. R., Groenink, W., van Koert, P., Schots, A., Bakker, J. & Smant, G. (2000). An efficient cDNA-AFLP-based strategy for the identification of putative pathogenicity factors from the potato cyst nematode *Globodera rostochiensis*. *Molecular Plant-Microbe Interactions* **13**, 830-836.

Qin, L. X. & Kerr, K. F. (2004). Empirical evaluation of data transformations and ranking statistics for microarray analysis. *Nucleic Acids Research* **32**, 5471-5479.

Rademaker, J. L. W., Hoste, B., Louws, F. J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P. & De Brujin, F. J. (2000). Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *International Journal of Systematic and Evolutionary Microbiology* **50**, 665-677.

Rademaker, J. L. W., Louws, F. J., Schultz, M. H., Rossbach, U., Vauterin, L., Swings, J. & de Bruijn, F. J. (2005). A comprehensive species to strain taxonomic framework for *Xanthomonas*. *Phytopathology* **95**, 1098-1111.

Raetz, C. R. H. & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annual Review of Biochemistry* **71**, 635-700.

Rafalski, A. (2002). Applications of single nucleotide polymorphisms in crop genetics. *Current Opinion In Plant Biology* **5**, 94-100.

Rajagopal, L., Sundari, C. S., Balasubramanian, D. & Sonti, R. V. (1997). The bacterial pigment xanthomonadin offers protection against photodamage. *FEBS Letters* **415**, 125-128.

Ramalingam, J., Cruz, C. M. V., Kukreja, K., Chittoor, J. M., Wu, J. L., Lee, S. W., Baraoidan, M., George, M. L., Cohen, M. B., Hulbert, S. H., Leach, J. E. & Leung, H. (2003). Candidate defense genes from rice, barley, and maize and their association with qualitative and quantitative resistance in rice. *Molecular Plant-Microbe Interactions* **16**, 14-24.

Raskin, D. M., Seshadri, R., Pukatzki, S. U. & Mekalanosl, J. J. (2006). Bacterial genomics and pathogen evolution. *Cell* **124**, 703-714.

Rechsteiner, M. P., Widmer, F. & Kölliker, R. (2006). Expression profiling of Italian ryegrass (*Lolium multiflorum* Lam.) during infection with the bacterial wilt inducing pathogen *Xanthomonas translucens* pv. *graminis*. *Plant Breed* **125**, 43-51.

Reheul, D., De Cauwer, B. & Cougnon, M. (2010). The role of forage crops in multifunctional agriculture. In *Fodder crops and amenity grasses*, pp. 1-12. Edited by B. Boller, U. K. Posselt & F. Veronesi: Springer.

Reidsma, P., Tekelenburg, T., van den Berg, M. & Alkemade, R. (2006). Impacts of land-use change on biodiversity: An assessment of agricultural biodiversity in the European Union. *Agriculture Ecosystems & Environment* **114**, 86-102.

Restrepo, S., Duque, M. C. & Verdier, V. (2000). Characterization of pathotypes among isolates of *Xanthomonas axonopodis* pv. *manihotis* in Colombia. *Plant Pathol* **49**, 680-687.

Rigano, L. A., Siciliano, F., Enrique, R., Sendin, L., Filippone, P., Torres, P. S., Questa, J., Dow, J. M., Castagnaro, A. P., Vojnov, A. A. & Marano, M. R. (2007). Biofilm formation, epiphytic fitness, and canker development in *Xanthomonas axonopodis* pv *citri*. *Molecular Plant-Microbe Interactions* **20**, 1222-1230.

Roden, J. A., Belt, B., Ross, J. B., Tachibana, T., Vargas, J. & Mudgett, M. B. (2004). A genetic screen to isolate type III effectors translocated into pepper cells during *Xanthomonas* infection. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **101**, 16624-16629.

Rodrigues, F. A., McNally, D. J., Datnoff, L. E., Jones, J. B., Labbe, C., Benhamou, N., Menzies, J. G. & Belanger, R. R. (2004). Silicon enhances the accumulation of diterpenoid phytoalexins in rice: A potential mechanism for blast resistance. *Phytopathology* **94**, 177-183.

Rott, P. & Davis, M. (2000). Leaf scald. In *A guide to sugarcane diseases*, pp. 339. Edited by P. Rott, R. Bailey, Comstock J., Croft B. & A. Saumtally. Montpellier.

Rozen, S. & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, pp. 365-386. Edited by S. Krawetz & S. Misener. Totowa, NJ: Humana Press.

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J. C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thebault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J. & Boucher, C. A. (2002). Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature* **415**, 497-502.

Salmeron, J. M., Oldroyd, G. E. D., Rommens, C. M. T., Scofield, S. R., Kim, H. S., Lavelle, D. T., Dahlbeck, D. & Staskawicz, B. J. (1996). Tomato *Prf* is a memeber of

leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. *Cell* **86**, 123-133.

Salzberg, S. L., Sommer, D. D., Schatz, M. C., Phillippy, A. M., Rabinowicz, P. D., Tsuge, S., Furutani, A., Ochiai, H., Delcher, A. L., Kelley, D., Madupu, R., Puiu, D., Radune, D., Shumway, M., Trapnell, C., Aparna, G., Jha, G., Pandey, A., Patil, P. B., Ishihara, H., Meyer, D. F., Szurek, B., Verdier, V., Koebnik, R., Dow, J. M., Ryan, R. P., Hirata, H., Tsuyumu, S., Lee, S. W., Ronald, P. C., Sonti, R. V., Van Sluys, M. A., Leach, J. E., White, F. F. & Bogdanove, A. J. (2008). Genome sequence and rapid evolution of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* PXO99(A). *BMC Genomics* **9**, 16.

Samudrala, R., Heffron, F. & McDermott, J. E. (2009). Accurate prediction of secreted substrates and identification of a conserved putative secretion signal for type III secretion systems. *PLoS Pathogens* **5**.

Sandkvist, M. (2001). Type II secretion and pathogenesis. *Infection and Immunity* **69**, 3523-3535.

Saraste, M., Sibbald, P. R. & Wittinghofer, A. (1990). The P-loop - A common motif in ATP-binding and GTP-binding proteins. *Trends In Biochemical Sciences* **15**, 430-434.

Schaad, N. W., Vidaver, A. K., Lacy, G. H., Rudolph, K. & Jones, J. B. (2000). Evaluation of proposed amended names of several pseudomonads and xanthomonads and recommendations. *Phytopathology* **90**, 208-213.

Scheideler, M., Schlaich, N. L., Fellenberg, K., Beissbarth, T., Hauser, N. C., Vingron, M., Slusarenko, A. J. & Hoheisel, J. D. (2002). Monitoring the switch from housekeeping to pathogen defense metabolism in *Arabidopsis thaliana* using cDNA arrays. *Journal Of Biological Chemistry* **277**, 10555-10561.

Schejbel, B., Jensen, L. B., Asp, T., Xing, Y. & Lübberstedt, T. (2008). Mapping of QTL for resistance to powdery mildew and resistance gene analogues in perennial ryegrass. *Plant Breed* **127**, 368-375.

Schmidt, D. (1988a). Bacterial wilt of forage grasses: strategies to limit disease dispearsal through mowing. *Revue Suisse d'agriculture* **20**, 351-357.

Schmidt, D. (1988b). Bacterial wilt of grasses: tests to limit the spread of the disease at harvest. *Revue Suisse d'agriculture* **20**, 351-357.

Schmidt, D. (1988c). Prevention of bacterial wilt of grasses by phylloplane bacteria. *Journal of Phytopathology* **122**, 253-260.

Schmidt, D. (1989). Epidemiological aspects of bacterial wilt of fodder grasses. *EPPO Bulletin* **19**, 89-95.

Schneider, S., Roessli, D. & Excoffier, L. (2000). *Arlequin: a software for population genetics data analysis*. Geneva, Switzerland: Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.

Schornack, S., Ballvora, A., Gürlebeck, D., Peart, J., Ganal, M., Baker, B., Bonas, U. & Lahaye, T. (2004). The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3. *Plant Journal* **37**, 46-60.

Schreiber, A. W., Sutton, T., Caldo, R. A., Kalashyan, E., Lovell, B., Mayo, G., Muehlbauer, G. J., Druka, A., Waugh, R., Wise, R. P., Langridge, P. & Baumann, U. (2009). Comparative transcriptomics in the *Triticeae*. *BMC Genomics* **10**, 17.

Schubiger, F. X., Hürlimann, H.-C., Schläppi, K., Streckeisen, P. & Boller, B. (2006). Virulence of crown rust isolates on genotypes of Italian and perennial ryegrass. *XXVI EUCARPIA Fodder Crops and Amenity Grasses Section Meeting, Perugia, 3-7 September*, 301-306.

Seong, E. S., Choi, D., Cho, H. S., Lim, C. K., Cho, H. J. & Wang, M. H. (2007). Characterization of a stress-responsive ankyrin repeat-containing zinc finger protein of *Capsicum annuum* (CaKR1). *Journal of Biochemistry and Molecular Biology* **40**, 952-958.

Severin, A. J., Woody, J. L., Bolon, Y. T., Joseph, B., Diers, B. W., Farmer, A. D., Muehlbauer, G. J., Nelson, R. T., Grant, D., Specht, J. E., Graham, M. A., Cannon, S. B., May, G. D., Vance, C. P. & Shoemaker, R. C. (2010). RNA-Seq atlas of glycine max: A guide to the soybean transcriptome. *BMC Plant Biology* **10**.

Siguier, P., Perochon, J., Lestrade, L., Mahillon, J. & Chandler, M. (2006). ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Research* **34**, D32-D36.

Silva, I. T., Rodrigues, F. A., Oliveira, J. R., Pereira, S. C., Andrade, C. C. L., Silveira, P. R. & Conciecao, M. M. (2010). Wheat resistance to bacterial leaf streak mediated by silicon. *Journal of Phytopathology* **158**, 253-262.

Smyth, G. K. (2005). *Limma: linear models for microarray data*. New York, NY.

Song, Y. L., Wang, G. L., Chen, L. L., Kim, H. S., Pi, L. Y., Holsten, T., Gardner, J., Wang, B., Zhai, W. X., Zhu, L. H., Fauquet, Z. & Ronald, P. (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science* **279**, 1804-1806.

Spoor, W. (1976). Self-incompatibility in *Lolium perenne* L. *Heredity* **37**, 417-421.

Starr, M. P. & Stephens, W. L. (1964). Pigmentation and taxonomy of the genus *Xanthomonas*. *Journal of Bacteriology* **87**, 293-302.

Stoodley, P., Sauer, K., Davies, D. G. & Costerton, J. W. (2002). Biofilms as complex differentiated communities. *Annual Review of Microbiology* **56**, 187-209.

Studer, B., Boller, B., Herrmann, D., Bauer, E., Posselt, U. K., Widmer, F. & Kölliker, R. (2006). Genetic mapping reveals a single major QTL for bacterial wilt resistance in Italian ryegrass (*Lolium multiflorum* Lam.). *Theoretical and Applied Genetics* **113**, 661-671.

Studer, B., Kölliker, R., Muylle, H., Asp, T., Frei, U., Roldán-Ruiz, I., Barre, P., Tomaszewski, C., Meally, H., Barth, S., Skøt, L., Armstead, I. P., Dolstra, O. & Lübberstedt, T. (2010). EST-derived SSR markers used as anchor loci for the construction of a consensus linkage map in ryegrass (*Lolium* spp.). *BMC Plant Biology* **10**.

Stuiver, M. H. & Custers, J. (2001). Engineering disease resistance in plants. *Nature* **411**, 865-868.

Stuthman, D. D., Leonard, K. J. & Miller-Garvin, J. (2007). Breeding crops for durable resistance to disease. In *Advances in Agronomy, Vol 95*, pp. 319-367.

Sun, X. L., Cao, Y. L., Yang, Z. F., Xu, C. G., Li, X. H., Wang, S. P. & Zhang, Q. F. (2004). *Xa26*, a gene conferring resistance to *Xanthomonas oryzae* pv. *oryzae* in rice, encodes an LRR receptor kinase-like protein. *Plant Journal* **37**, 517-527.

Suter, D., Briner, H., Mosimann, E. & Jeangros, B. (2005). Sortenversuche mit Italienischem und Bastard-Raigras. *Agrarforschung* **12**, 242-247.

Sutherland, I. W. (2001). Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology-Uk* **147**, 3-9.

Swings, J. G. & Civerolo, E. L. (1993). *Xanthomonas*, First edn: Chapman & Hall.

Swords, K. M. M., Dahlbeck, D., Kearney, B., Roy, M. & Staskawicz, B. J. (1996). Spontaneous and induced mutations in a single open reading frame alter both virulence and avirulence in *Xanthomonas campestris* pv. *vesicatoria* avrBs2. *Journal of Bacteriology* **178**, 4661-4669.

Tang, D., Wu, W., Li, W., Lu, H. & Worland, A. J. (2000). Mapping of QTLs conferring resistance to bacterial leaf streak in rice. *Theoretical and Applied Genetics* **101**, 286-291.

Tanksley, S. D. (1993). Mapping polygenes. *Annual Review of Genetics* **27**, 205-233.

Tao, Y., Xie, Z. Y., Chen, W. Q., Glazebrook, J., Chang, H. S., Han, B., Zhu, T., Zou, G. Z. & Katagiri, F. (2003). Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell* **15**, 317-330.

The R Development Core Team (2008).R: a language and environment for statistical computing. In *R Foundation for Statistical Computing*. Vienna, Austria.

Thieme, F., Koebnik, R., Bekel, T., Berger, C., Boch, J., Buttner, D., Caldana, C., Gaigalat, L., Goesmann, A., Kay, S., Kirchner, O., Lanz, C., Linke, B., McHardy, A. C., Meyer, F., Mittenhuber, G., Nies, D. H., Niesbach-Klosgen, U., Patschkowski, T., Ruckert, C., Rupp, O., Schneiker, S., Schuster, S. C., Vorhölter, F. J., Weber, E., Pühler, A., Bonas, U., Bartels, D. & Kaiser, O. (2005). Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* revealed by the complete genome sequence. *Journal of Bacteriology* **187**, 7254-7266.

Thieme, F., Szczesny, R., Urban, A., Kirchner, O., Hause, G. & Bonas, U. (2007). New type III effectors from *Xanthomonas campestris* pv. *vesicatoria* trigger plant reactions dependent on a conserved N-myristoylation motif. *Molecular Plant-Microbe Interactions* **20**, 1250-1261.

Thorogood, D., Paget, M., Humphreys, M., Turner, L., Armstead, I. & Roderick, H. (2001). QTL analysis of crown rust resistance in perennial ryegrass - implications for breeding. *International Turfgrass Society Research Journal* **9**, 218-223.

Tibshirani, R. (1996). Regression shrinkage and selection via the LASSO. *Journal of the Royal Statistical Society Series B-Methodological* **58**, 267-288.

Tilquin, P. (2003). Methodological aspects of the mapping of disease resistance loci in live-stock: Université catholique de Louvain.

Torres, P. S., Malamud, F., Rigano, L. A., Russo, D. M., Marano, M. R., Castagnaro, A. P., Zorreguieta, A., Bouarab, K., Dow, J. M. & Vojnov, A. A. (2007). Controlled synthesis of the DSF cell-cell signal is required for biofilm formation and virulence in *Xanthomonas campestris*. *Environmental Microbiology* **9**, 2101-2109.

Valasek, M. A. & Repa, J. J. (2005). The power of real-time PCR. *Advances in Physiology Education* **29**, 151-159.

Van Treuren, R., Bas, N., Goossens, P. J., Jansen, J. & Van Soest, L. J. M. (2005). Genetic diversity in perennial ryegrass and white clover among old Dutch grasslands as compared to cultivars and nature reserves. *Mol Ecol* **14**, 39-52.

Vauterin, L., Hoste, B., Kersters, K. & Swings, J. (1995). Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology* **45**, 472-489.

Vauterin, L., Rademaker, J. & Swings, J. (2000). Synopsis on the taxonomy of the genus *Xanthomonas*. *Phytopathology* **90**, 677-682.

Velculescu, V. E., Zhang, L., Vogelstein, B. & Kinzler, K. W. (1995). Serial analysis of gene expression. *Science* **270**, 484-487.

Verdier, V., Restrepo, S., Mosquera, G., Jorge, V. & Lopez, C. (2004). Recent progress in the characterization of molecular determinants in the *Xanthomonas axonopodis* pv. *manihotis*-cassava interaction. *Plant Molecular Biology* **56**, 573-584.

Vorhölter, F. J., Schneiker, S., Goesmann, A., Krause, L., Bekel, T., Kaiser, O., Linke, B., Patschkowski, T., Rueckert, C., Schmid, J., Sidhu, V. K., Sieber, V., Tauch, A., Watt, S. A., Weisshaar, B., Becker, A., Niehaus, K. & Puehler, A. (2008). The genome of *Xanthomonas campestris* pv. *campestris* B 100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthesis. *Journal of Biotechnology* **134**, 33-45.

Vorhölter, F. J., Niehaus, K. & Pühler, A. (2001). Lipopolysaccharide biosynthesis in *Xanthomonas campestris* pv. *campestris*: a cluster of 15 genes is involved in the biosynthesis of the LPS O-antigen and the LPS core. *Molecular Genetics and Genomics* **266**, 79-95.

Wan, J., Dunning, F. & Bent, A. (2002). Probing plant-pathogen interactions and downstream defense signaling using DNA microarrays. *Functional and Integrative Genomics* **2**, 259-273.

Wang, G. L., Song, W. Y., Ruan, D. L., Sideris, S. & Ronald, P. C. (1996). The cloned gene, *Xa21*, confers resistance to multiple *Xanthomonas oryzae* pv. *oryzae* isolates in transgenic plants. *Molecular Plant-Microbe Interactions* **9**, 850-855.

Wang, H. M. & Sletten, A. (1995). Infection biology of bacterial wilt of forage grasses. *Journal of Phytopathology* **143**, 141-145.

Wang, K., Kang, L., Anand, A., Lazarovits, G. & Mysore, K. S. (2007). Monitoring in planta bacterial infection at both cellular and whole-plant levels using the green fluorescent protein variant GFPuv. *New Phytologist* **174**, 212-223.

Wang, L. F., Rong, W. & He, C. Z. (2008). Two *Xanthomonas* extracellular polygalacturonases, PghAxc and PghBxc, are regulated by type III secretion regulators HrpX and HrpG and are required for virulence. *Molecular Plant-Microbe Interactions* **21**, 555-563.

Wang, Z., Gerstein, M. & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics* **10**, 57-63.

Weber, M., Harada, E., Vess, C., von Roepenack-Lahaye, E. & Clemens, S. (2004). Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant Journal* **37**, 269-281.

Wei, C. F., Kvitko, B. H., Shimizu, R., Crabill, E., Alfano, J. R., Lin, N. C., Martin, G. B., Huang, H. C. & Collmer, A. (2007). A *Pseudomonas syringae* pv. *tomato* DC3000 mutant lacking the type III effector HopQ1-1 is able to cause disease in the model plant *Nicotiana benthamiana*. *Plant Journal* **51**, 32-46.

Wei, Z. M., Sneath, B. J. & Beer, S. V. (1992). Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. *Journal of Bacteriology* **174**, 1875-1882.

Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D. & McClelland, M. (1992). Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Research* **20**, 4965-4970.

Wengelnik, K., VandenAckerveken, G. & Bonas, U. (1996). HrpG, a key hrp regulatory protein of *Xanthomonas campestris* pv. *vesicatoria* is homologous to two-component response regulators. *Molecular Plant-Microbe Interactions* **9**, 704-712.

Wengelnik, K., Rossier, O. & Bonas, U. (1999). Mutations in the regulatory gene *hrpG* of *Xanthomonas campestris* pv. *vesicatoria* result in constitutive expression of all *hrp* genes. *Journal of Bacteriology* **181**, 6828-6831.

Whalen, M. C., Wang, J. F., Carland, F. M., Heiskell, M. E., Dahlbeck, D., Minsavage, G. V., Jones, J. B., Scott, J. W., Stall, R. E. & Staskawicz, B. J. (1993). Avirulence gene *avrRxv* from *Xanthomonas campestris* pv. *vesicatoria* specifies resistance on tomato line Hawaii-7998. *Molecular Plant-Microbe Interactions* **6**, 616-627.

Whalen, M. C., Richter, T., Zakhareyvich, K., Yoshikawa, M., Al-Azzeh, D., Adefioye, A., Spicer, G., Mendoza, L. L., Morales, C. Q., Klassen, V., Perez-Baron, G., Toebe, C. S., Tzovolous, A., Gerstman, E., Evans, E., Thompson, C., Lopez, M. & Ronald, P. C. (2008). Identification of a host 14-3-3 protein that interacts with *Xanthomonas* effector AvrRxv. *Physiological and Molecular Plant Pathology* **72**, 46-55.

White, F., Potnis, N., Jones, J. & Koebnik, R. (2009). The type III effectors of *Xanthomonas*. *Mol Plant Pathol* **10**, 749-766.

White, F. F., Johnson, L. B. & Yang, B. (2000). Prospects for understanding avirulence gene function. *Current Opinion in Plant Biology* **3**, 291-298.

White, F. F. & Yang, B. (2009). Host and pathogen factors controlling the rice-*Xanthomonas oryzae* interaction. *Plant Physiology* **150**, 1677-1686.

Wichmann, F., Müller-Hug, B., Widmer, F., Boller, B., Studer, B. & Kölliker, R. (2010). Phenotypic and molecular characterization indicate no major race-specific interactions between *Xanthomonas translucens* pv. *graminis* and *Lolium multiflorum*. *Plant Pathol* **published ahead of print 2010 Oct 11.**

Wilkins, P. W. (1975). Inheritance of resistance to *Puccinia coronata* corda and *Rhynchosporium orthosporum* caldwell in Italian ryegrass. *Euphytica* **24**, 191-196.

Wilkins, P. W. (1978). Specialization of crown rust (*Puccinia coronata* corda) on clones of Italian ryegrass (*Lolium multiflorum* Lam.). *Euphytica* **27**, 837-841.

Wilkins, P. W. & Humphreys, M. O. (2003). Progress in breeding perennial forage grasses for temperate agriculture. *Journal of Agricultural Science* **140**, 129-150.

Wise, R. P., Moscou, M. J., Bogdanove, A. J. & Whitham, S. A. (2007). Transcript profiling in host-pathogen interactions. *Annual Review of Phytopathology* **45**, 329-369.

Wojtaszek, P. (1997). Oxidative burst: An early plant response to pathogen infection. *Biochemical Journal* **322**, 681-692.

Woo, E. J., Dunwell, J. M., Goodenough, P. W., Marvier, A. C. & Pickersgill, R. W. (2000). Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nature Structural Biology* **7**, 1036-1040.

Wright, S. Y., Suner, M. M., Bell, P. J., Vaudin, M. & Greenland, A. J. (1993). Isolation and characterization of male flower cDNAs from maize. *Plant Journal* **3**, 41-49.

Wu, J. L., Sinha, P. K., Variar, M., Zheng, K. L., Leach, J. E., Courtois, B. & Leung, H. (2004). Association between molecular markers and blast resistance in an advanced backcross population of rice. *Theoretical and Applied Genetics* **108**, 1024-1032.

Wydra, K., Zinsou, V., Jorge, V. & Verdier, V. (2004). Identification of pathotypes of *Xanthomonas axonopodis* pv. *manihotis* in Africa and detection of quantitative trait loci and markers for resistance to bacterial blight of cassava. *Phytopathology* **94**, 1084-1093.

Yamazaki, A., Hirata, H. & Tsuyumu, S. (2008). HrpG regulates type II secretory proteins in *Xanthomonas axonopodis* pv. *citri*. *J Gen Plant Pathol* **74**, 138-150.

Yan, W. & Falk, D. E. (2002). Biplot analysis of host-by-pathogen data. *Plant Disease* **86**, 1396-1401.

Yang, B., Sugio, A. & White, F. F. (2006). *Os8N3* is a host disease-susceptibility gene for bacterial blight of rice. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 10503-10508.

Yang, W. C. & Francis, D. M. (2005). Marker-assisted selection for combining resistance to bacterial spot and bacterial speck in tomato. *Journal of the American Society for Horticultural Science* **130**, 716-721.

Yang, Y. H. & Speed, T. (2002). Design issues for cDNA microarray experiments. *Nature Reviews Genetics* **3**, 579-588.

Yang, Z., Sun, X., Wang, S. & Zhang, Q. (2003). Genetic and physical mapping of a new gene for bacterial blight resistance in rice. *Theor Appl Genet* **106**, 1467.

Yoshimura, S., Yamanouchi, U., Katayose, Y., Toki, S., Wang, Z. X., Kono, I., Kurata, N., Yano, M., Iwata, N. & Sasaki, T. (1998). Expression of *Xa1*, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 1663-1668.

Young, J. M., Dye, D. W., Bradbury, J. F., Panagopoulos, C. G. & Robbs, C. F. (1978). Proposed nomenclature and classification for plant pathogenic bacteria. *New Zealand Journal of Agricultural Research* **21**, 153-177.

Yuan, M., Chu, Z. H., Li, X. H., Xu, C. G. & Wang, S. P. (2009). Pathogen-induced expressional loss of function is the key factor in race-specific bacterial resistance conferred by a recessive *R*-gene *xa13* in rice. *Plant Cell Physiol* **50**, 947-955.

Zhang, G. J., Guo, G. W., Hu, X. D., Zhang, Y., Li, Q. Y., Li, R. Q., Zhuang, R. H., Lu, Z. K., He, Z. Q., Fang, X. D., Chen, L., Tian, W., Tao, Y., Kristiansen, K., Zhang, X. Q., Li, S. G., Yang, H. M. & Wang, J. (2010). Deep RNA sequencing at single base-pair resolution reveals high complexity of the rice transcriptome. *Genome Research* **20**, 646-654.

Zhang, S. S., He, Y. Q., Xu, L. M., Chen, B. W., Jiang, B. L., Liao, J., Cao, J. R., Liu, D., Huang, Y. Q., Liang, X. X., Tang, D. J., Lu, G. T. & Tang, J. L. (2008). A putative

colR(XC1049)-col(XC1050) two-component signal transduction system in *Xanthomonas campestris* positively regulates *hrpC* and *hrpE* operons and is involved in virulence, the hypersensitive response and tolerance to various stresses. *Research in Microbiology* **159**, 569-578.

Zhao, B. Y., Ardales, E. Y., Raymundo, A., Bai, J. F., Trick, H. N., Leach, J. E. & Hulbert, S. H. (2004). The *avrRxo1* gene from the rice pathogen *Xanthomonas oryzae* pv. *oryzicola* confers a nonhost defense reaction on maize with resistance gene *Rxo1*. *Molecular Plant-Microbe Interactions* **17**, 771-779.

Zhu, Y. Y., Chen, H. R., Fan, J. H., Wang, Y. Y., Li, Y., Chen, J. B., Fan, J. X., Yang, S. S., Hu, L. P., Leung, H., Mew, T. W., Teng, P. S., Wang, Z. H. & Mundt, C. C. (2000). Genetic diversity and disease control in rice. *Nature* **406**, 718-722.

Zou, L. F., Wang, X. P., Xiang, Y., Zhang, B., Li, Y. R., Xiao, Y. L., Wang, J. S., Walmsley, A. R. & Chen, G. Y. (2006). Elucidation of the hrp clusters of *Xanthomonas oryzae* pv. *oryzicola* that control the hypersensitive response in nonhost tobacco and pathogenicity in susceptible host rice. *Applied and Environmental Microbiology* **72**, 6212-6224.

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9 Curriculum vitae

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Education

2007-2011 **Ph.D. thesis**
Molecular Ecology group, Agroscope Reckenholz-Tänikon Research Station ART, Zürich, Switzerland
Supervisor: Dr. Roland Kölliker
Committee members: Prof. Beat Keller, Prof. Robert Dudler
Title: "Genetic characterization of the interaction between Italian ryegrass (*Lolium multiflorum* Lam.) and *Xanthomonas translucens* pv. *graminis*"

2008 **PhD exchange with Det Jordbrugvidenskabelige Fakultaet (DJF, Aarhus University, Denmark)**
6 month of exchange within the framework of the PhD project

2004 - 2006 **Graduate studies**
Awarded M.Sc. ETH in Agricultural Engineering
Plant Biotechnology group, Swiss Federal Institute of Technology (ETH) Zürich, Switzerland.

Supervisors: Dr. Christof Sautter, Dr. Bartoz Urbaniak
Title: "*Ustilago tritici* resistance and expression profile of transgenic KP4 wheat"

2001 - 2004 **Undergraduate studies**
Major studies in Agrobiotechnology
Minor studies in Crop Science
Swiss Federal Institute of Technology Zürich (ETH), Switzerland

1997 - 2000 **Matura Type B** (university-entrance diploma)
Gymnasium Münchenstein, Münchenstein, Switzerland

Research interests

- **Plant–pathogen interactions**
- **Plant breeding**
- **Functional genomics**

Laboratory experiences

- **DNA and RNA work:** PCR, sequencing, cloning, Southern blotting, DNA preparation, microarray target preparation and hybridization, Real-time PCR, SSR markers, etc.
- **Plant and cell work:** infection of plants, isolation of bacteria from plant material, transformation of bacteria

Experience in bioinformatics

- **Programming and statistical analyses:** microarray analysis with R
- **Sequence analysis:** Blast, VectorNTI, BioEdit, multiple sequence alignments (ClustalW), genome/gene annotation software GenDB, MEGA4

Training

2010 **Introduction to GenDB for annotation of bacterial genomes**, Centre for Biotechnology (CeBiTec), Bielefeld, Germany

2009 **Molecular biology of plant-pathogen interactions**, University of Zürich, Switzerland

2008 **Statistical methods for gene expression analyses**, University of Zürich, Switzerland
Semester course including exercises in R

Scientific writing I + II, University of Zürich, Switzerland
Two courses including exercises

2006 **Microarray Data Analysis**
Workshop including exercises in R, Swiss Federal Institute of Technology Lausanne (EPFL), Switzerland

Teaching experience

2009 **Supervision of two bachelor theses**
Molecular Ecology group, Agroscope Reckenholz-Tänikon Research Station ART, Zürich, Switzerland

Assistant and tutor for 2nd year undergraduate students in practical course "Plant biology", assisting students during three weeks
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Oral presentations

2011 Presentation at the "**4th Swiss Microbial Ecology Meeting", Engelberg, Switzerland**

Title: "Identification of virulence-associated genes in *Xanthomonas translucens* pv. *graminis*"

2010 Presentation at the "**6th Symposium for Molecular Breeding of Forage and Turf" (MBFT2010), Buenos Aires, Argentina**
Title: "Identification of genes involved in bacterial wilt resistance of *Lolium multiflorum*"

2009 Presentation at the "**5th Annual Symposium of PhD students of Agroscope" in Tänikon, CH**
Title: "Identification of genes induced in *Lolium multiflorum* by bacterial wilt infection"

Poster presentations

2009 Poster at the "**EUCARPIA: 28th Meeting of the Fodder Crops and Amenity Grasses Section", La Rochelle, France**
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2008 Poster at the "**7th Plant Genomics European Meeting" (Plant GEM), Albena, Bulgaria**
Title: "Identification of genes in *Lolium multiflorum* differentially expressed upon infection with *Xanthomonas translucens* pv. *graminis*"

Awards

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Publications

1. **Fabienne Wichmann**, Bernhard Müller Hug, Franco Widmer, Beat Boller, Bruno Studer, Roland Kölliker. **"Phenotypic and molecular genetic characterization indicate no major race-specific interactions between *Xanthomonas translucens* pv. *graminis* and *Lolium multiflorum*."(2011)** Plant Pathology 60 (2) 314-324

2. **Fabienne Wichmann**, Torben Asp, Franco Widmer, Roland Kölliker. **"Transcriptional responses of Italian ryegrass during interaction with *Xanthomonas translucens* pv. *graminis* reveal novel candidate genes for bacterial wilt resistance." (2011)** Theoretical and Applied Genetics 122 (3) 567-579.

3. **Fabienne Wichmann**, Franco Widmer, Roland Kölliker. **"The *hrpG* gene of *Xanthomonas translucens* pv. *graminis* contributes to symptom development during bacterial wilt infection but is not essential for *in planta* survival."** in preparation